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Major Sclerotial Polypeptides Of Psychrophilic Fungi: Identification, Immunological Relatedness, Localization And In Vivo Synthesis

Wayne Jay Newsted

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MAJOR SCLEROTIAL POLYPEPTIDES OF PSYCHROPHILIC FUNGI: IDENTIFICATION, IMMUNOLOGICAL RELATEDNESS, LOCALIZATION AND *IN VIVO* SYNTHESIS

by

Wayne Jay Newsted

Department of Plant Sciences

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ABSTRACT

Major sclerotial polypeptides have been identified by one and two-dimensional gel electrophoresis in Myriosclerotinia borealis, Coprinus psychromorbidus, Typhula idahoensis and Typhula incarnata. The number, molecular mass, relative proportions and isoelectric points of the major sclerotial polypeptides varied from species to species. Furthermore, several of the major sclerotial polypeptides were shown to be antigenically related by Ouchterlony double-diffusion and Western blot analysis. Polyclonal antibodies used for localizing the major sclerotial polypeptides in the sclerotia by immunofluorescence microscopy showed that the major polypeptides were sequestered in sclerotial protein bodies in all four species. This indicates that the major sclerotial polypeptides may play a role as storage proteins. Major sclerotial polypeptides were not detected in vegetative hyphae of M. borealis and C. psychromorbidus at 5°C, but significant accumulations were observed at 25°C by Western blot analysis. Major sclerotial polypeptides were detected in the vegetative hyphae of the Typhula species incubated at 5°C. Fluorographic analysis combined with Western blotting showed that synthesis of the major sclerotial polypeptides was induced when vegetative hyphae of M. borealis and C. psychromorbidus were shifted from 5°C to 10°C, whereas vegetative hyphae of T. idahoensis and T.

incarnata showed constituitive synthesis of some of the major sclerotial polypeptides at 5°C. A shift from 5°C to 10°C had little effect on the synthesis of major sclerotial polypeptides in the *Typhula* spp. Thus, vegetative hyphae of *M. borealis* and *C. psychromorbidus* appeared to be much more sensitive to temperature as a cue for inducing synthesis of the major sclerotial polypeptides than vegetative hyphae of the *Typhula* spp. The significance of these observations and their bearing on sclerotial development in the species examined is discussed.

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ABBREVIATIONS

Anti LRS131	Antisera raised to the major sclerotial polypeptides of *Coprinus psychromorbidus* LRS131.
Anti W21	Antisera raised to the major sclerotial polypeptides of *T. idahoensis* (W21).
Anti W29	Antisera raised to the major sclerotia polypeptides of *T. incarnata* (W29).
Anti W51	Antisera raised to the major sclerotial polypeptides of *M. borealis* (W51).
BSA	bovine serum albumin
BSM	basic synthetic medium
c.g.	carpogenic germination
Ci	Curie
cpm	counts per minute
EDTA	ethylenediamine tetraacetic acid
FITC	fluoroscein isothiocynate
GAR	goat anti-rabbit
HRP	horseradish peroxidase
IEF	isoelectric focussing
IgG	gamma globulin
kD	kilodalton

LRS131	*Coprinus psychromorbidus* isolate LRS131
mA	milliampere
m.g.	myceliogenic germination
Mr	molecular mass
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
Tris	tris-hydroxymethylaminomethane
V	volt
W21	*Typhula idahoensis* isolate W21
W29	*Typhula incarnata* isolate W29
W51	*Myriosclerotinia borealis* isolate W51

CHAPTER 1

GENERAL INTRODUCTION

1.1 CLASSIFICATION AND PATHOGENICITY OF SNOW MOLDS

Collectively, snow molds represent a diverse taxonomic group and belong to a broad spectrum of fungal genera and phyla (Smith, 1981; Jamalainen, 1974). Major ascomycetous snow molds include Gerlachia nivalis (Ces.ex.Sacc.) W. Gams and E. Muller var. nivalis (Smith, 1981) and Myriosclerotinia borealis (Bub. and Vleug.) Kohn (Smith 1981; Kohn, 1979). The principal basidiomycetous snow molds are Typhula idahoensis Remsb. (Bruehl, 1982), Typhula incarnata Remsb. (Bruehl, 1982), and Coprinus psychromorbidus Redhead and Traquair (Redhead and Traquair, 1981). M. borealis, C. psychromorbidus, T. idahoensis and T. incarnata have been reported as severe pathogens of winter wheat and overwintering turfgrasses (Scott, 1957; Bruehl and Cunfer, 1971; Traquair, 1980; Smith, 1981; Bruehl, 1982). In fact, all four of these snow molds are capable of parasitizing graminaceous plants under low temperature conditions (Jamalainen, 1974) and all four can form dormant over-summering structures called sclerotia (Fig. 1). Though most snow molds form sclerotia under the appropriate conditions, not all of them have this capability (Smith, 1981). The following study is

restricted to the examination of the sclerotial-forming snow molds, M. borealis, C. psychromorbidus, T. idahoensis and T. incarnata.

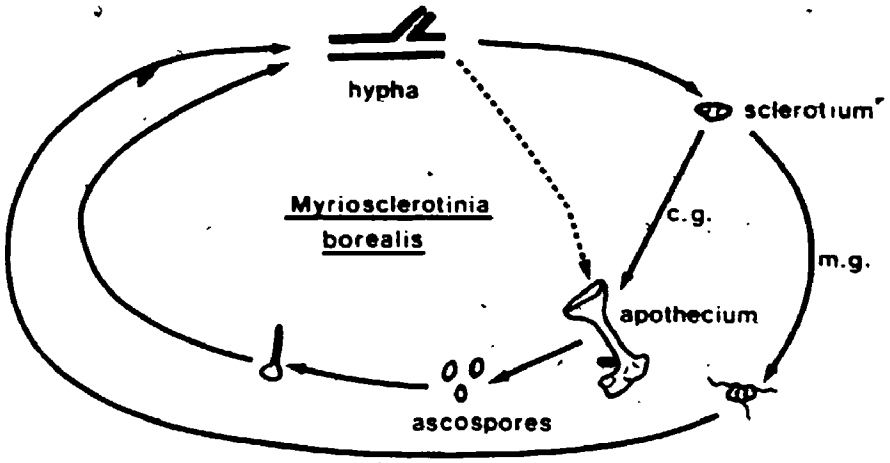
1.2 LIFE CYCLES OF SNOW MOLDS

Though they differ taxonomically, M. borealis, C. psychromorbidus, T. idahoensis and T. incarnata have very similar life cycles. The main difference lies in the type of sexual fruiting structure and spores they produce. All of these fungi are capable of forming sclerotia (Fig. 1) which are asexual, multicellular, resting structures (Chet and Henis, 1975). These structures are the means by which a quiescent viable state is maintained in the absence of a suitable host or when adverse growing conditions prevail (Coley-Smith and Cooke, 1971). Conditions favouring sclerotia formation are usually prevalent in the spring, just after snow melt (Smith, 1981). An interesting feature of sclerotia is that they can germinate via two different and mutually exclusive mechanisms, one producing infectious vegetative hyphae, that is, myceliogenic germination (Fig. 1 m.g.), the other producing sexual fruiting bodies (apothecia in M. borealis, mushrooms in C. psychromorbidus and sporophores in the Typhula species). This is referred to as carpogenic germination (Fig. 1 c.g.) (Russo et al., 1982).

Both types of germination usually occur in the fall

Figure 1. A summary of the life cycles of M. borealis, C. psychromorbidus and the Typhula spp. Dashed lines indicate events which have not been documented and may not occur in nature. The letters c.g. and m.g. designate carpogenic and myceliogenic germination respectively.

SNOW MOLD LIFE CYCLES



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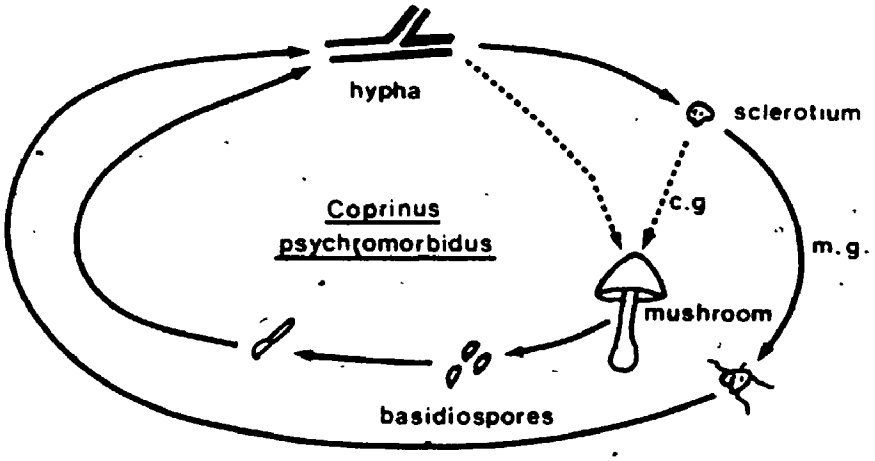
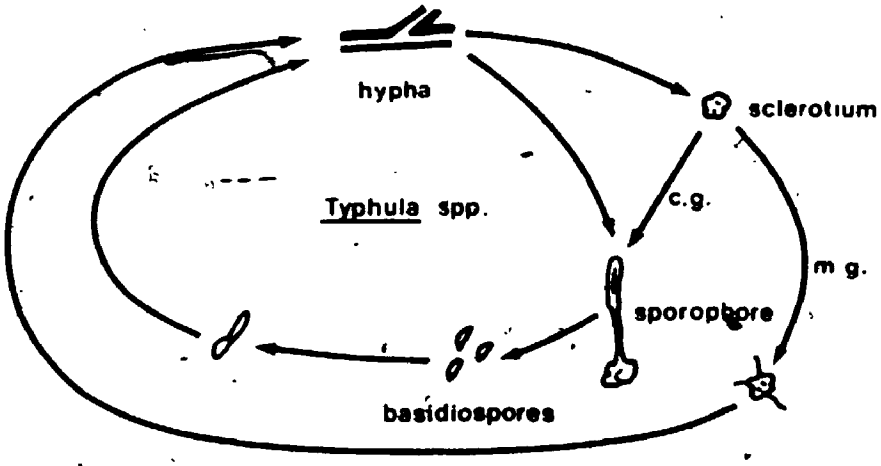


Adopted from Traquair, J. A. 1980.



Adopted from Remsberg, R.E. 1940.

when cool moist conditions prevail (Smith, 1981). To germinate carpogenically, however, mature sclerotia must undergo physiological conditioning (Russo et al., 1982). Several factors, including temperature and light, influence such conditioning (Coley-Smith and Cooke, 1971). The end result of carpogenic germination is the production of sexual fruiting bodies which produce spores; ascospores in M. borealis and basidiospores in C. psychromorbidus and the Typhula spp (Fig. 1). These spores can then germinate to form vegetative hyphae which can grow, infect and destroy plants at subzero temperatures (Jamalainen, 1974). Alternatively, hyphae produced myceliogenically or from germinated spores can undergo sclerotial morphogenesis (Fig. 1) to complete the cycle.

1.3 STUDIES OF OTHER PSYCHROPHILIC ORGANISMS

Most progress in the area of the physiology and biochemistry of psychrophily has come from the studies of other microorganisms, including psychrophilic yeasts and bacteria (Scott, 1957; Ingraham and Stokes, 1959; Hagen and Rose, 1961; Somkuti, 1980). Hagen and Rose (1961) were able to show that Cryptococcus, a psychrophilic yeast, was unable to grow at 30°C (mesophilic range), but resumed growth after it was returned to cold (permissive) temperature. From their work, they deduced two possible explanations for the biochemical basis of psychrophilic

behaviour in microorganisms. First, they suggested that at higher temperatures, obligate psychrophiles may produce toxic compounds that inactivate certain detoxifying enzymes. The alternative explanation was that psychrophilic behaviour in microorganisms may be a reflection of thermal inactivation of certain specific enzymes. Malcolm (1968a, 1968b, 1969) showed that psychrophily in Micrococcus cryophilus appeared to be directly correlated to the temperature sensitivity of specific amino acid activating enzymes. More recently, Innis (1975) noted the advantage of using temperature-sensitive mutants to gain an understanding of the mechanisms by which temperature exerts an effect on a psychrophilic organism. He also observed that since the ability of psychrophiles to grow at low temperature can be altered by mutation, then such ability probably depends on the properties of various proteins. However, Somkuti (1980) recently presented evidence that the DNA and ribosomes of thermophilic fungi were much more heat-stable than the DNA and ribosomes of psychrophilic fungi, thus implicating these cellular components as molecular determinants of thermophily and psychrophily. Though these studies present possible mechanisms for psychrophily, it is unknown whether these mechanisms are biochemically operative in snow mold fungi.

1.4 PHYSIOLOGY AND BIOCHEMISTRY OF SNOW MOLDS

To date, the majority of snow mold studies can be classified as epidemiological, descriptive or taxonomic in nature (Newsted et al., 1985). An exception to this was the physiological work of Ward and coworkers (Ward, 1968a, 1968b; Dejardin and Ward, 1971a, 1971b).

Ward (1968a) reported evidence for the uncoupling of growth from respiration in M. borealis (syn. Sclerotinia borealis) at supramaximal temperatures and suggested that "some mechanism for the disposal of energy-rich products must be postulated." In another paper, Ward (1968b) also demonstrated that exposure to supramaximal growth temperatures induced changes in the hyphal morphology of M. borealis. He found that vegetative growth of M. borealis was arrested at 20°C and that an immediate increase in the diameter of the hyphae and a loss of hyalinity occurred. Furthermore, peripheral hyphae of a colony shrivelled, but older hyphae continued to thicken. He also found that a resumption of growth at the optimum temperature occurred from these older hyphae.

Data on the growth and respiration of Typhula species have also been published by Dejardin and Ward (1971a, 1971b). In their first paper, Dejardin and Ward (1971a) reported that the level of endogenous respiration in T. idahoensis was very high. In their subsequent paper, Dejardin and Ward (1971b) demonstrated that endogenous

respiration was stimulated by glucose at 5°C and 20°C and by acetate at 5°C. Furthermore, the extent of this stimulation was generally increased by starvation of the mycelium.

Nonpermissive temperature, by definition, represents the growth-restricting temperature above which the organism ceases to grow vegetatively (Malcolm, 1968a). Recently, Newsted et al. (1985) studied the effects of temperature on the vegetative growth and polypeptide composition of several snow mold species.

Nonpermissive temperatures were determined by allowing vegetative hyphae of each species to grow at permissive temperature (4°C). After maximum steady-state rates of growth were observed, cultures were shifted to nonpermissive temperatures for varying periods of time. Upon shifting to nonpermissive temperatures, vegetative growth was immediately arrested. When cultures were shifted back to permissive temperature, growth rates comparable to control rates were observed. M. borealis (W51) was shown to have a nonpermissive temperature of 22°C, whereas the nonpermissive temperatures for two Typhula spp. and two Coprinus spp. were determined to be in the 25°C-30°C range. In a similar manner, Ward (1971) was able to demonstrate nonpermissive growth temperatures in a basidiomycetous snow mold. Furthermore, Newsted et al. (1985) demonstrated that a general breakdown of most polypeptides occurred when vegetative hyphae of M. borealis

(W51) and the Typhula species were subjected to supra-maximal growth temperatures. However, polypeptides of molecular mass similar to the major sclerotial polypeptides were evident.

Earlier, Ward (1968a) had reported the uncoupling of growth from respiration in M. borealis at nonpermissive growth temperatures. Newsted et al. (1985) subsequently postulated that the uncoupling of growth from respiration may "reflect an attempt to change developmentally from a mycelial growth form to a sclerotial growth form." This postulate was based on two observations. First, when hyphae of M. borealis and both Typhula species were subjected to nonpermissive temperature, the polypeptides that prevailed were of a molecular mass comparable to those observed in mature sclerotia of these snow molds (Insell et al., 1985) even though sclerotia were not visible on the plates. Second, Dejardin and Ward (1971a) reported that sclerotial production on undefined medium was stimulated when T. incarnata was shifted to nonpermissive temperature.

Very recently, Traquair et al. (1987) reported the effects of temperature on the in vitro production of sclerotia in C. psychromorbidus. They found that the optimum temperatures for sclerotium production in all isolates of C. psychromorbidus were higher than the optimum temperatures for mycelial growth. Thus, their results indicated that higher temperatures play a role in stimulating sclerotial morphogenesis. This report

represents one of the few recent studies on the physiology of sclerotial morphogenesis in snow molds. The relevance of their findings corroborates observations made in this thesis.

1.5 STUDIES ON THE PHYSIOLOGY, BIOCHEMISTRY AND DEVELOPMENTAL BIOLOGY OF OTHER FUNGI

A considerable amount of recent data has accumulated on the biochemistry and the molecular biology of development in mesophilic sclerotial-forming fungi (Petersen et al., 1982; Russo et al., 1982). Russo et al. (1982) presented evidence that sclerotia of Sclerotinia sclerotiorum (a mesophile) contained large quantities of a stage specific protein (SSP) that was either lacking or present in low amounts in vegetative hyphae. They were able to demonstrate by one-dimensional SDS-PAGE that SSP had an apparent molecular weight of 33,000 and comprised about 38% of the total sclerotial protein. SSP did not appear to be a glycoprotein due to the lack of staining with Schiff reagent. Examination by two-dimensional gel electrophoresis showed that a majority of SSP migrated as a single polypeptide with an isoelectric point of 6.0. However, they found that two other minor bands with pI values of 5.8 and 6.2 also had molecular weights similar to SSP. Furthermore, they observed that the protein appeared to be development or stage specific, since it accumulated rapidly during sclerotial formation. Two days after the

initiation of sclerotial formation, SSP already comprised 25% of the total sclerotial protein. By 21 days, SSP comprised 30% of the total sclerotial protein. They suggested that the protein could accumulate via two mechanisms. It could be synthesized at a more rapid rate than the other proteins or else it could be more resistant to degradation than the other sclerotial proteins. The first possibility was favoured since the major product formed from in vitro translation of polysomal RNA isolated from young sclerotia is the sclerotial protein (Russo et al., 1982).

In a later paper, Petersen et al. (1982) compared proteins extracted from the sclerotia of Sclerotinia trifoliorum, Sclerotinia minor and Sclerotinia sclerotiorum. They found that sclerotia of all three species contained a major protein. In addition, these major proteins appeared to be immunologically similar but they differed in their molecular weights and isoelectric points (Petersen et al., 1982). However, it is perplexing that Petersen et al. (1982) failed to detect a major sclerotial polypeptide in Sclerotiorum rolfsii, since Insell et al. (1985) clearly demonstrated the presence of a major polypeptide of approximately 16 kD. Insell et al. (1985) suggested that Petersen et al. (1982) overlooked this major sclerotial polypeptide because of the polyacrylamide gradient employed during electrophoretic separation.

Other results from the paper of Petersen et al. (1982) seemed to indicate that the major sclerotial proteins may somehow act as a storage protein, that is, as sources of amino acids during carpogenic germination. This idea was supported by the work of Russo et al. (1982) where they showed electrophoretically that a protein of molecular weight similar to SSP also accumulated in the stipe of apothecia. Further evidence in this regard was published by Russo and Van Etten (1985) where they used an immunoelectron microscopic technique to show that SSP accumulated in protein bodies which resembled those found in the seeds of higher plants.

Moore and Jirjis (1981) performed electrophoretic studies of carpophore development in the basidiomycete, Coprinus cinereus, and found that the protein pattern in the cap was always different from that found in the stipe. Thus, they demonstrated that proteins associated with various developmental structures can be quite different.

Very recently, Silva et al. (1987) have observed changes in the pattern of protein synthesis during zoospore germination in Blastocladiella emersonii. They found that protein synthesis was regulated at various levels of gene expression during zoospore germination. Transcriptional, translational and post-translational controls were clearly implicated.

In another series of studies, Bullock et al. (1980a, 1980b, 1983), examining the ultrastructure of Sclerotinia

minor, showed evidence of protein bodies in the cortical and medullary hyphae of the sclerotia. Russo and Van Etten (1985) localized the major sclerotial protein of S. sclerotiorum in protein bodies using immunogold labelling.

1.6 PROTEIN BODIES IN SEEDS

It has been shown in mesophilic sclerotial-forming fungi (Russo et al., 1982) and in psychrophilic sclerotial-forming fungi (Chapter 2) that there is an accumulation of major proteins as a function of sclerotial development. Furthermore, Russo and Van Etten (1985) have shown that major sclerotial proteins of S. sclerotiorum accumulate in organelles which morphologically resemble protein bodies in the seeds of higher plants. Although protein bodies are commonly found in seeds of plants (Lott, 1980; Spitzer and Lott, 1982), there are only a few reports of such organelles in fungi. Thus, to gain an understanding of protein body structure, composition, and development, it is necessary to review the literature of seed protein bodies. Such an investigation should prove informative since plant seeds and fungal sclerotia represent analogous perennating structures.

A majority of scientific literature on plant protein deposits involve the study of seed protein bodies (Lott, 1980). Protein bodies in seeds are of great importance to most plants because they provide nitrogenous compounds and

minerals necessary for the establishment of plant seedlings. This is analogous to carpogenic germination of sclerotia in fungi. Though this section will deal only with seed protein bodies, it is important to realize that proteinaceous deposits have also been reported in nonseed tissues such as roots (Peumans et al., 1984), leaves (Shumway et al., 1970), stems (Kidwai and Robards, 1969), flowers (Shumway et al., 1972) and bark (Greenwood et al., 1985). Generally, protein bodies in seeds appear to have a limiting membrane and can have different ultrastructural morphologies: amorphous, crystalloid, globoids or rosette crystals (Lott, 1980).

Studies of the development of protein bodies in plant seeds seem to indicate that protein bodies can form in different ways (Lott, 1980; Chrispeels et al., 1982; Akazawa and Hara-Nishimura, 1985). One mechanism suggests a vacuolar origin whereas another favours the concept of protein body formation from specialized regions of endoplasmic reticulum or from small cytoplasmic vesicles (Chrispeels et al., 1982; Akazawa and Hara-Nishimura, 1985). Numerous reports support both mechanisms (Kyle and Styles, 1977; Chrispeels, 1983, 1985). It is probable that similar mechanisms exist for protein body formation in the psychrophilic sclerotial-forming fungi.

In contrast, published information concerning the synthesis of major storage proteins in fungal sclerotia is sparse. This is not surprising since there are few papers

published on the biochemistry of sclerotial development. One exception, however, is the paper by Russo and Van Etten (1985) where the synthesis of a development-specific protein in sclerotia of Sclerotinia sclerotiorum was examined. They suspected that SSP (development-specific sclerotial protein), like many storage proteins in seeds, would be synthesized as a precursor protein, that is, have a signal sequence which is cleaved to yield mature SSP during transport into these protein bodies. However, attempts to detect such a precursor failed. They concluded that SSP is probably a member of the rare group of exported proteins which lack a cleavable signal sequence. The question still remains as to the mechanism of synthesis of the major sclerotial polypeptides in snow molds. Though this question will not be addressed in this thesis, studies reported in later chapters should help lay the foundation for future investigations.

1.7 HEAT SHOCK PROTEINS IN FUNGI

Temperature has been reported as an important stimulus of sclerotial development in C. psychromorbidus (LRS131) (Traquair et al., 1987). Furthermore, changes in gene expression associated with sclerotial development have been demonstrated in other fungi (Russo et al., 1982). Therefore, a brief review of the literature on heat shock proteins in fungi is discussed.

Several years ago it was discovered that heat shock led to the new and/or enhanced synthesis of a characteristic set of polypeptides in Drosophila melanogaster (Tissières et al., 1974). This observation has spawned extensive investigations into the genetic and molecular events associated with the response to heat shock in many other organisms. The heat shock response has now been recorded in nearly all organisms, from bacteria to plants and animals (Schlesinger et al., 1982). However, compared to other organisms, the study of the heat shock response in fungi has begun very recently and has been documented in only a few species including Saccharomyces cerevisiae (McAlister et al., 1979), Physarum polycephalum (Wright and Tollon, 1982), Dictyostelium (Loomis and Wheeler, 1980), Neurospora crassa (Michèa-Hamzehpour et al., 1980) and Achlya (Silver et al., 1983). Thus far, the heat shock response has not been studied in psychrophilic sclerotial forming fungi.

Generally, the heat shock response to elevated temperature is characterized by the rapid and massive synthesis of a few "heat shock" proteins of varying molecular masses against a background of reduced "normal" protein synthesis (Plesofsky-Vig and Brambl, 1985a). The heat shock response in fungi is similar in many respects to that observed in other organisms. However, in most studies the fungal response has been shown to be quite transient even though the fungi are maintained at high temperature

(McAlister et al., 1979; Silver et al., 1983; Plesofsky-Vig and Brambl, 1985b). Typically, the response lasts for 30 to 60 minutes before returning to normal protein synthesis while the fungus remains at high temperature. Thus, fungi differ from animals which are generally unable to synthesize normal proteins during exposure to heat shock (Atkinson, 1981), and they differ from plants that require several hours or more to recover normal protein synthesis at high temperature (Key et al., 1981). The brevity of the heat shock response in fungi may be related to the cellular mode of respiration because Lindquist et al. (1982) have shown that S. cerevisiae cells that were utilizing fermentative respiration had a short-lived heat shock response, whereas cells provided with a non fermentable carbon source (e.g., acetate) used oxidative respiration and showed a prolonged heat shock response. Therefore, access to an alternate mode of respiration may be the explanation for the short-lived heat shock response in fungi. Whether all fungi elicit an ephemeral heat shock response is unknown.

Regulation of heat shock protein synthesis has been shown to occur at various levels of gene expression. In most organisms, synthesis of heat shock proteins requires new transcription (Plesofsky-Vig and Brambl, 1985a). However, post-transcriptional control of preferred heat shock protein synthesis in S. cerevisiae cells has been demonstrated (Miller et al., 1979). They found that normal

mRNAs were degraded at an accelerated rate during heat shock. In addition, translational control of heat shock has been established in the conidiospores of Neurospora crassa (Plesofsky-Vig and Brambl, 1985b). They observed that heat shock mRNAs were preserved in the conidiospores and were translated into heat shock proteins during the first hour of incubation at high temperature. Therefore, it appears that multiple levels of regulation are possible in heat shock protein synthesis in fungi.

Numerous functions of heat shock proteins have been proposed. Several studies have shown a direct relationship between synthesis of heat shock proteins and acquisition of thermotolerance (McAlister and Finkelstein, 1980; Plesofsky-Vig and Brambl, 1985b). However, other agents such as ethanol (Plesset et al., 1982), hormones (Ireland and Berger, 1982) and metallic ions (Lindquist, 1986) have also been shown to induce heat shock protein synthesis rendering cells resistant to lethal temperatures. Consequently, it is possible that many of the heat shock proteins are not specifically involved in thermotolerance, since heat shock protein synthesis is a general response that is induced by several stimuli (Plesofsky-Vig and Brambl, 1985a).

Heat shock proteins may also play a role in growth arrest, although it is uncertain whether heat shock directly induces growth arrest resulting in characteristic proteins or whether the proteins themselves induce growth

arrest (Iida and Yahara, 1984). One of the more plausible explanations for the existence of heat shock proteins is that they are proteins synthesized as a result of developmental change. For example, Kurtz and Linquist (1984) extracted RNA at different times from diploid S. cerevisiae cells that were growing in a sporulation medium. They found three distinct phases of RNA synthesis. During the first phase, there was a decrease in the mRNAs common during vegetative growth and an induction of mRNAs for two heat shock proteins. The two subsequent phases of mRNA expression involved the induction of RNAs that later disappeared and were succeeded by a group of RNAs that were stored in the dormant spores. Furthermore, the preservation of the heat shock RNAs in the mature spores indicated that the heat shock RNAs may play a role in the developmental process of sporulation. Recently, it has also been shown that during the transition from vegetative growth to differentiation that D. discoideum produces mRNAs that encode heat shock proteins (Zuker et al., 1983).

In regard to the functions of heat shock proteins, it is very perplexing that few studies of heat shock protein synthesis have been coupled with studies on the localization of the heat shock proteins in the cells. Localizing these proteins in the cells could very well improve the current understanding of the role of heat shock proteins (Plesofsky-Vig and Brambl, 1985a).

1.8 PROPOSED RESEARCH AND THESIS OBJECTIVES

In this study, the primary focus is to examine major proteins associated with sclerotial development in the four psychrophilic fungi, Myriosclerotinia borealis, Coprinus psychromorbidus, Typhula idahoensis and Typhula incarnata.

The major objectives of this thesis are as follows: a) to provide a preliminary identification and characterization of proteins specifically associated with sclerotial development using one and two-dimensional gel electrophoresis in the four sclerotial forming snow molds; and b) to isolate, purify and raise antibodies to any major proteins associated with sclerotial development. (c) The antibodies are used to determine the antigenic relatedness of these proteins via Ouchterlony double-diffusion and Western blotting; and d) to localize these proteins in the sclerotial cells via immunofluorescence. These data may provide useful information in determining a probable function of these proteins. The final objectives of this thesis examine the relationship between environmental temperature and the expression of the major sclerotial proteins. (e) Western blotting is used to probe for the accumulation of the major sclerotial polypeptides in vegetative hyphae incubated at permissive and nonpermissive temperatures; and lastly, f) fluorographic analysis coupled with Western blotting is used to examine the temperature-induced synthesis of the major sclerotial polypeptides in

vegetative hyphae of the four psychrophilic fungi subjected to different temperature treatments.

CHAPTER 2

MAJOR POLYPEPTIDES ASSOCIATED WITH SCLEROTIAL DEVELOPMENT IN FOUR SNOW MOLDS

2.1 INTRODUCTION

Studies presented in this chapter examine for the first time, polypeptide compositions associated with sclerotial development in the four psychrophilic fungi, Myriosclerotinia borealis (W51), Coprinus psychromorbidus (LRS131), Typhula idahoensis (W21) and Typhula incarnata (W29). Sclerotial development is divided into three distinct morphological states: vegetative hyphae, sclerotial initials, an intermediate stage characterized by white to flesh-coloured hyphal tufts, and mature sclerotia (Figs. 2-5). This nomenclature is similar to that previously adopted by Bullock et al. (1980) in their histochemical study of sclerotial development in Sclerotinia minor, a mesophilic sclerotial-forming fungus.

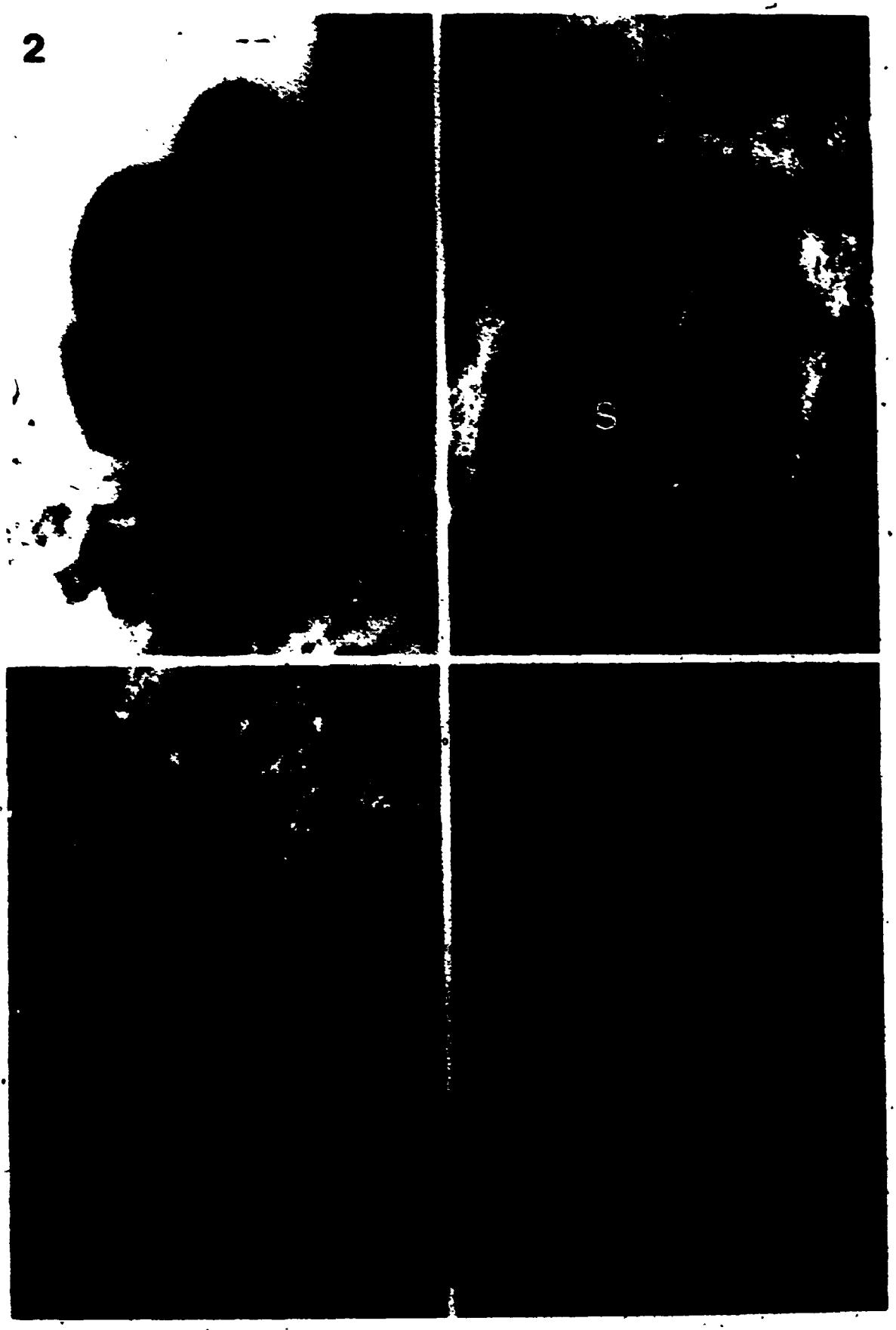
One-dimensional gel electrophoresis is used to characterize developmentally dependent changes in the polypeptide profiles which accompany sclerotial development and presents the first evidence for the existence of major sclerotial polypeptides in these fungi. Two-dimensional gel electrophoresis is used to measure the isoelectric points of the major sclerotial polypeptides. In M.

Figure 2. Photograph of mature sclerotia of M. borealis (W51). Mature sclerotia (S). Bar equals 1 mm.

Figure 3. Photograph of mature sclerotia of C. psychromorbidus (LRS131). Mature sclerotia (S). Bar equals 1 mm.

Figure 4. Photograph of mature sclerotia of T. idahoensis (W21). Mature sclerotia (S). Bar equals 1 mm.

Figure 5. Photograph of mature sclerotia of T. incarnata (W29). Mature sclerotia (S). Bar equals 1 mm.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borealis, evidence that the major sclerotial polypeptides exist in nature is furnished by comparing polypeptide profiles of field grown and cultured sclerotia. In addition, the sensitivity of the major sclerotial polypeptides of the two Typhula species to different denaturing conditions is also examined. Collectively, these studies provide the initial characterization of the major sclerotial polypeptides and form the foundation for subsequent investigations.

2.2 MATERIALS AND METHODS

2.2.1 Cultures.

Myriosclerotinia borealis (W51), Typhula idahoensis (W21) and Typhula incarnata (W29) were obtained from E.W.B. Ward (Agriculture Canada, London, Ontario). Coprinus psychromorbidus (LRS131) was provided by J.A. Traquair (Harrow Research Station, Harrow, Ontario). Field sclerotia from Alaska were supplied by M. Griffith (University of Waterloo, Waterloo, Ontario).

2.2.2 Growth conditions.

Cultures of Myriosclerotinia borealis (W51), Coprinus psychromorbidus (LRS131) Typhula idahoensis (W21) and Typhula incarnata (W29) were grown in darkness at 5°C in petri dishes on a defined agar medium containing Difco Bacto-agar, 17.0 g; dextrose, 15.0 g; L-asparagine, 2.36 g;

KH_2PO_4, 1.0 g; $MgSO_4$ (anhydrous), 0.24 g; and minor nutrients (See Appendix I) and vitamins (See Appendix II) brought to 1 L with double distilled water (pH 6.5). Cultures grown in this manner routinely produced vegetative hyphae and sclerotial initials in 2-3 weeks. Mature sclerotia were formed in 4-6 weeks (Figs. 2-5).

2.2.3 Protein extraction.

Vegetative hyphae were collected from the surface of the agar using forceps. After the hyphae were removed, sclerotial initials and mature sclerotia were collected by gently scraping the surface of the agar. Vegetative hyphae, sclerotial initials and mature sclerotia were pulverized in separate mortars with pestles at 0°-4°C in cold extraction buffer (pH 6.8) containing 0.05 M Tris, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% (v/v) ß-mercaptoethanol and proteolytic inhibitors including 6 mM 4-aminobenzamidine dihydrochloride, 40 mM 6-aminocaproic acid and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged for 10 min. at 9,000 xg at 4°C. The pellets were discarded and the supernatants were extracted with an equal volume of chloroform:methanol (24:1). Protein which precipitated at the interface was carefully removed and dissolved in cold extraction buffer. Small aliquots (100 µl) from each protein solution were set aside for total protein determination using the Bio-Rad protein assay according to Bradford (1976) (see Appendix

III). An equal volume of cold methanol was then added to the remainder of the protein solutions to precipitate the protein. The suspensions were centrifuged for 10 minutes at 9,000 xg at 4°C, the supernatants were discarded, and the protein precipitates were immediately prepared for solubilization.

2.2.4 Protein solubilization.

Protein samples to be used for one-dimensional SDS polyacrylamide slab gel electrophoresis were solubilized in a minimal amount of an SDS sample buffer (pH 6.8) containing 0.5 M Tris-HCl, 1% (w/v) SDS, 0.2 M EDTA, 1% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and proteolytic inhibitors including 6 mM p-aminobenzamidine, 40 mM 6-aminocaproic acid and 1 mM PMSF. Sclerotial protein samples to be used for two-dimensional electrophoresis were solubilized in a urea sample buffer containing 9.5 M urea, 2% (w/v) Nonidet P40, 5% (w/v) β-mercaptoethanol and ampholines (LKB) including 1% (w/v) pH 3.5-10 ampholine, 0.7% (w/v) pH 5-7 ampholine and 0.3% (w/v) pH 9-11 ampholine. All samples were stored at -70°C.

The following method was used to determine whether the denaturant used prior to electrophoresis had a significant effect on the polypeptide patterns observed. Sclerotial protein of W21 and W29 was extracted as previously described and solubilized in the following buffers: (i) SDS buffer only (ii) initially in SDS sample buffer but

with the subsequent addition of urea to a final concentration of 4.25 M and (iii) initially in urea sample buffer (minus ampholines) with the subsequent addition of SDS to a final concentration of 2.3% (w/v). Protein concentrations were determined by the turbidimetric assay of Comings and Tack (1972) (see Appendix IV) using 16.6% (w/v) trichloracetic acid as precipitant.

2.2.5 One-dimensional SDS polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide gel electrophoresis was performed using modifications of the procedure described by Laemmli (1970).

Separating gels were cast using a two-chambered gradient maker (Chrismac Plastic Fabrications). For maximum resolution, separating gels (pH 8.8) consisting of the following polyacrylamide gradients were used: 12-18% (w/v) acrylamide for W51 and W29; 18-28% (w/v) acrylamide for LRS131 and 15-22% (w/v) acrylamide for W21. Separating gels were allowed to polymerize for at least 1 hour.

Stacking gels (pH 6.8) consisting of 5% (w/v) acrylamide were poured on top of the separating gel and allowed to polymerize for 30 minutes. The running buffer consisted of 0.05 M Tris-HCl (pH 8.3), 0.4 M glycine, 1% (w/v) SDS and 0.2 M EDTA. Thawed protein samples were prepared by adding approximately 2-3 µl of 0.5% (w/v) bromophenol blue to the protein solutions and then boiling the solutions for 1 minute. Approximately 50 µg of protein were loaded into preformed wells in the stacking gel. In

addition, a 4 µl sample of standard proteins was loaded onto one well for relative molecular mass (Mr) determination of resolved polypeptides in each sample. Samples were electrophoresed on a Bio-Rad Protean dual vertical slab gel electrophoresis cell at a constant current of 12.5 mA per gel for 1 hour and then at 25 mA per gel until the dye front reached the bottom of the gel. At this point, gels were removed from the plates and placed in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol: 10% (v/v) acetic acid for 1 hour on a rotary shaker to simultaneously stain and fix the proteins. The gels were then destained in 40% (v/v) methanol: 7.5% (v/v) acetic acid on a rotary shaker until no background stain remained. The polypeptide profiles were then compared and photographed.

2.2.6 Gel scans.

Spectrophotometric scans of polypeptide profiles were obtained with a Shimadzu Graphicord UV-250 recording spectrophotometer fitted with a GSC-3 gel scanner and connected to a Shimadzu PR-1 graphic printer. The gels were scanned at 555 nm. The relative content of sclerotial polypeptides was estimated from peak areas of recorded scans using a LI-COR portable area meter (Model LI-3000, Lambda Instruments). Relative molecular mass was calculated using a Hewlett-Packard HP-85 computer.

2.2.7 Two-dimensional IEF-SDS-PAGE separations of sclerotial polypeptides.

Two-dimensional electrophoresis was based on the method of O-Farrell (1975). The first dimension isoelectric focussing (IEF) gel consisted of 3.8% (w/v) acrylamide, LKB ampholines including 1% (w/v) pH 3.5-10 ampholine, 0.7% (w/v) pH 5-7 ampholine and 0.3% (w/v) pH 9-11 ampholine, 2% (w/v) Nonidet P40 and 9.16 M urea. The lower reservoir contained 0.09 M orthophosphoric acid and the upper reservoir contained 0.1 M NaOH. Sclerotial protein samples (100 µg), solubilized in urea buffer as described above, were loaded onto gels that were prerun at 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 30 minutes. IEF was carried out at 400 V constant voltage for 12 hours followed by 1 hour at 800 V. Samples containing only urea lysis buffer were also electrofocussed in order to measure the pH gradient established in the gel. The pH gradient was measured using a Fisher Flat-Surface Combination Electrode (See Appendix V).

After electrofocussing, gels containing the sclerotial polypeptides were equilibrated for 1 hour in an equilibration buffer containing 0.063 M Tris, 2.3% (w/v) SDS, 10% (w/v) glycerol and 5% (w/v) ß-mercaptoethanol (pH 6.8). The equilibrated gels were secured to the top of gradient slab gels using 1% (w/v) agarose. Electrophoresis was carried out at 25 mA per gel constant current until the bromophenol blue dye front ran off the bottom of the gel. The gels were stained and destained as previously

described.

2.2.8 Photography of stained gels.

Stained gels were photographed on a piece of white plexiglass with background illumination. Kodak 2415 Technical Pan film was used in a 35 mm camera equipped with a yellow (No.8) Wratten filter to maximize contrast and resolution.

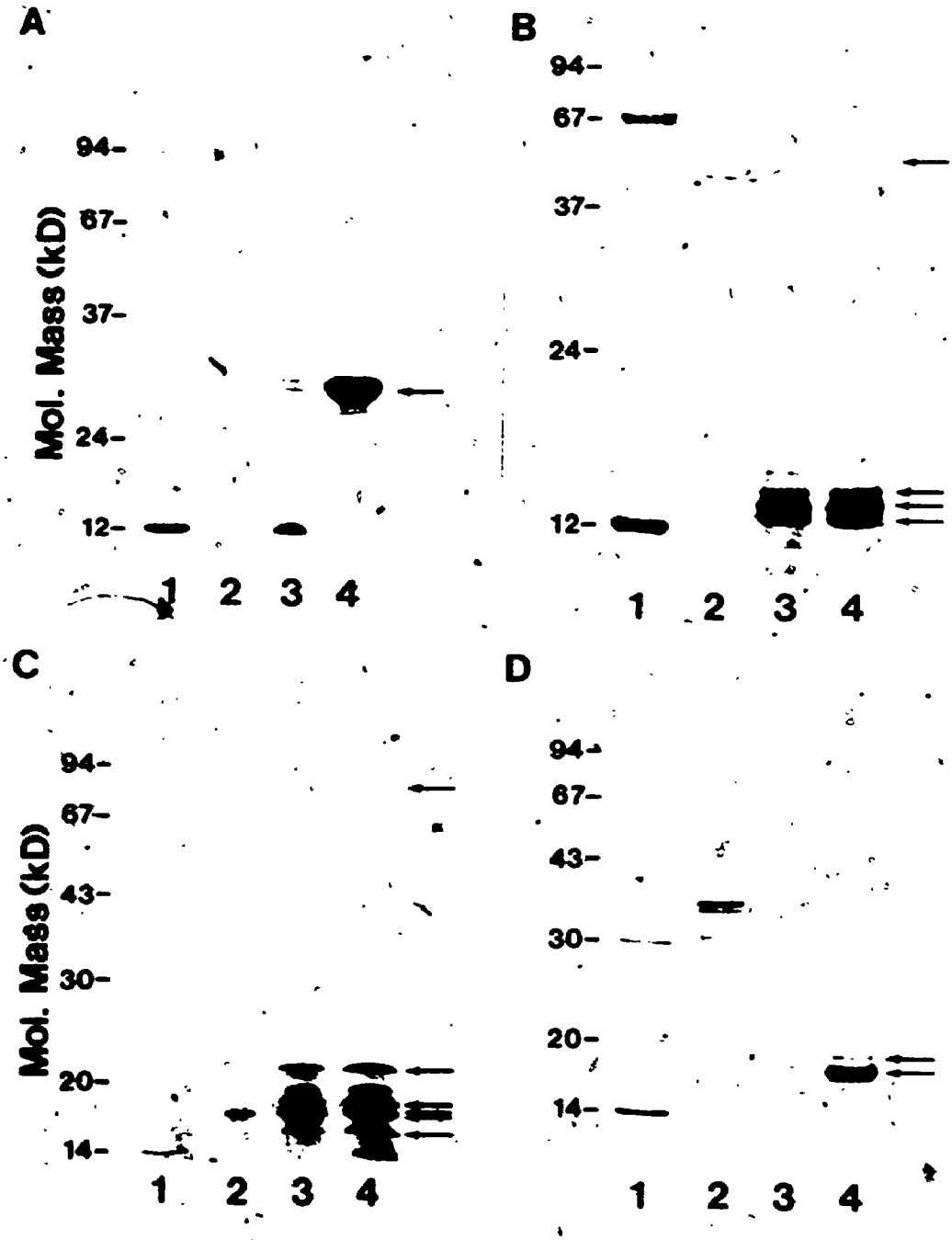
2.3 RESULTS

2.3.1 One-dimensional SDS-PAGE.

All snow mold species examined showed an apparent accumulation of major polypeptides. However, the number of polypeptides observed and their respective molecular weights varied from species to species. Myriosclerotinia borealis (W51) produced in culture showed an accumulation of a major polypeptide of approximately 33.9 kD (Fig. 6A, lane 4, arrow) constituting 30% (Table I) of the total sclerotial polypeptide complement. A polypeptide of similar molecular mass (≈33.9 kD) was discernible, however less prominent, in the sclerotial initials (Fig. 6A, lane 3) but was not apparent in the vegetative hyphae of W51 (Fig. 6A, lane 2).

Coprinus psychromorbidus (LRS131) exhibited an apparent accumulation of 3 major sclerotial polypeptides (Fig. 6B, lane 4, lower arrows) with molecular masses of 12.9, 13.8

Figure 6. Collage of one-dimensional SDS polyacrylamide gels. Lanes 1, standard proteins; lanes 2, vegetative hyphae; lanes 3, sclerotial initials; lanes 4, mature sclerotia. A. Polypeptide profiles of M. borealis (W51). Arrow indicates the major 33.9 kD sclerotial polypeptide. B. Polypeptide profiles of C. psychromorbidus (LRS131). Upper arrow indicates the 53.7 kD sclerotial polypeptide. Lower arrows in descending order indicate the 14.5, 13.8 and 12.9 kD sclerotial polypeptides. C. Polypeptide profiles of T. idahoensis (W21). Upper arrow indicates the 72.4 kD sclerotial polypeptide. Lower arrows in descending order indicate the 21.9, 18.2, 17.4, 17.0 and 16.2 kD sclerotial polypeptides. D. Polypeptide profiles of T. incarnata (W29). Arrows in descending order indicate the 18.6 kD and 17.0 kD sclerotial polypeptides. Molecular standards: phosphorylase b, 94 kD; bovine serum albumin, 67 kD; ovalbumin, 43 kD; alcohol dehydrogenase, 37 kD; carbonic anhydrase, 30 kD; chymotrypsinogen A, 24 kD; soybean trypsin inhibitor, 20 kD; α-lactalbumin, 14 kD and cytochrome c, 12 kD.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Mol. Mass (kD)
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Table I. Molecular masses of major polypeptides associated with sclerotial development in *M. borealis*, *C. psychromorbidus*, *T. idahoensis* and *T. incarnata*. Relative proportions refer only to major polypeptides of mature sclerotia.

Species	Molecular mass (kD) of major polypeptides: Sclerotial initials	Mature sclerotia	Relative proportion (% of total)
M. borealis	33.9[a]	33.9	30
C. psychromorbidus	12.9	12.9	
	13.8	13.8	51
	14.5	14.5	
	N[b]	53.9	4
T. idahoensis	N	16.2	
	17.0	17.0	
	17.4	17.4	48
	18.2	18.2	
	N	21.9	
	72.4	72.4	0.5
T. incarnata	N	17.0	60
	N	18.6	

[a] Percent error in molecular mass determinations was <10%.

[b] N, not apparent.

and 14.5 kD respectively. These polypeptides constituted about 51% (Table I) of the total sclerotial polypeptide complement. Polypeptides of similar molecular mass were also present in the sclerotial initials (Fig. 6B, lane 3) but were not apparent in the vegetative hyphae (Fig. 6B, lane 2). In addition, a major polypeptide of approximately 53.7 kD also appeared to accumulate in the sclerotia of LRS131 (Fig. 6B, lane 4, upper arrow) and constituted about 4% (Table I) of the total sclerotial polypeptide complement. However, this higher molecular weight polypeptide was not apparent in the vegetative hyphae or sclerotial initials (Fig. 6B, lanes 2 and 3).

Typhula idahoensis (W21) showed an apparent accumulation of at least 6 major sclerotial polypeptides. The molecular masses of the five low molecular mass polypeptides were 16.2, 17.0, 17.4, 18.2 and 21.9 kD respectively (Fig. 6C, lane 4, lower arrrows). These polypeptides constituted about 48% (Table I) of the total sclerotial polypeptide complement. A sixth major polypeptide of approximately 72.4 kD (Fig. 6C, lane 4, upper arrow) was also present and constituted 0.5% of the total sclerotial polypeptide complement (Table I). Four polypeptides of molecular mass similar to the low molecular mass polypeptides observed in the mature sclerotia were also apparent in the sclerotial initials of W21 (Fig. 6C, lane 3). These polypeptides included the 16.2, 17.0, 18.2 and 21.9 kD moieties. The 17.4 kD polypeptide was not

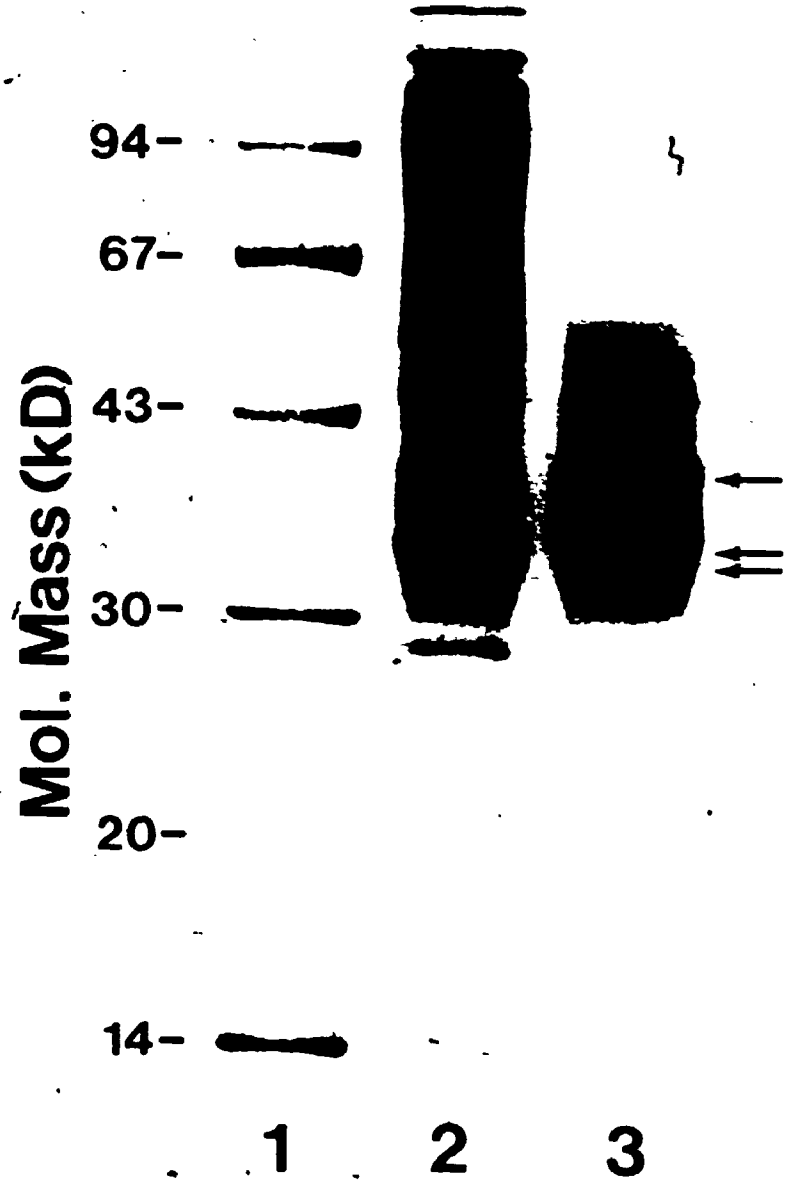
apparent in the sclerotial initials of W21 (Fig. 6C, lane 3). A polypeptide of similar molecular mass to the 72.4 kD polypeptide of mature sclerotia was also present and more prominent in the sclerotial initials than in the mature sclerotia (Fig. 6C, lane 3). Several polypeptides of molecular mass similar to the major low molecular weight sclerotial polypeptides were only barely discernible in the vegetative hyphae, but the high molecular weight moiety was not evident.

Typhula incarnata (W29) showed an apparent accumulation of one major sclerotial polypeptide of approximately 17.0 kD and a minor polypeptide of approximately 18.6 kD (Fig. 6D, lane 4, arrows). Together these polypeptides constituted about 60% of the total sclerotial polypeptide complement (Table I). Polypeptides of similar molecular mass were barely discernible in the sclerotial initials (Fig. 6D, lane 3) but were not apparent in the vegetative hyphae (Fig. 6D, lane 2).

2.3.2 Polypeptide profiles of cultured versus field grown sclerotia of M. borealis.

Comparison of the polypeptide profile of W51 sclerotia produced in culture with the polypeptide profile of sclerotia of M. borealis collected from the field indicated that the field sclerotia also contained a major polypeptide of about 33.9 kD (Fig. 7, lane 3, middle arrow). This polypeptide constituted about 42% of the total polypeptide complement in the field grown sclerotia whereas the 33.9 kD

Figure 7. One-dimensional SDS polyacrylamide gel of polypeptides from cultured and field grown sclerotia of M. borealis. Lane 1, standard proteins; lane 2, cultured sclerotia; lane 3, field sclerotia. Arrows in descending order indicate the 39.8, 33.9 and 32.4 kD polypeptides associated with field grown sclerotia. Molecular standards were: phosphorylase b, 94 kD; bovine serum albumin, 67 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD, soybean trypsin inhibitor, 20 kD and α-lactalbumin, 14 kD.


94-
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Mol. Mass (kD)
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polypeptide comprised only 20% of the total polypeptide complement in cultured sclerotia (Table II). Field sclerotia also appeared to contain considerable amounts of two other polypeptides, one of about 32.4 kD and the other of about 39.8 kD (Fig. 7, lane 3, lower and upper arrows respectively). Generally, fewer polypeptides were apparent in the field sclerotia of M. borealis compared to W51 sclerotia produced in culture (Fig. 7). Polypeptides of less than 30 kD were quite sparse in the field sclerotia, whereas numerous low molecular mass polypeptides persisted in W51 sclerotia formed in culture (Fig. 7).

2.3.3 Two-dimensional electrophoresis of sclerotial polypeptides.

In general, two-dimensional electrophoretic separations increased the resolution of sclerotial polypeptide complements of all species examined. This can be observed by comparing the one-dimensional separation in lanes 4 of Fig. 6 with their respective two-dimensional separation in Fig. 8.

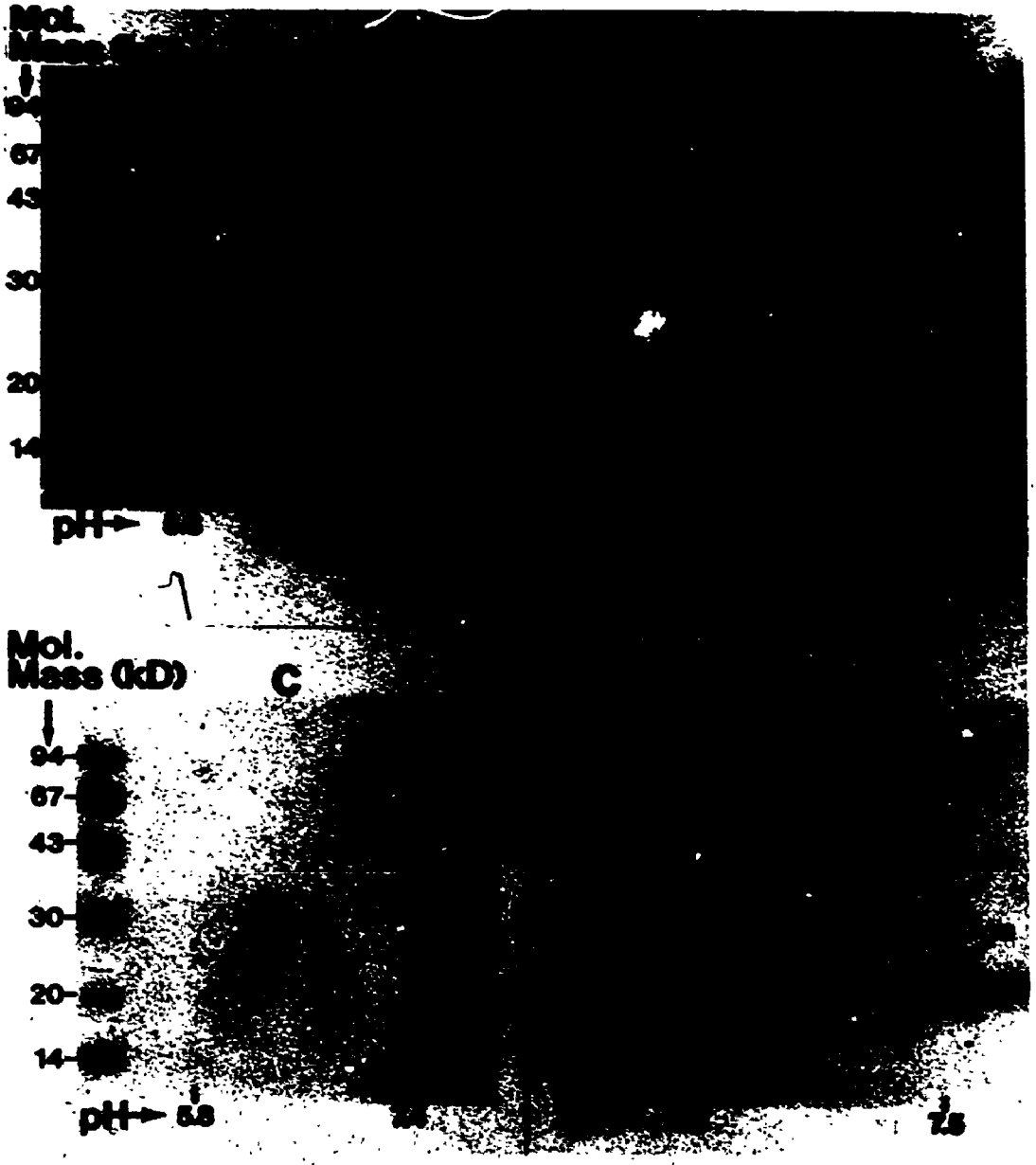
The single major sclerotial polypeptide of M. borealis (W51), observed after one dimensional SDS-PAGE, was resolved into two polypeptides of similar molecular mass but different isoelectric points. These polypeptides exhibited pI values of approximately 6.5 and 6.7 (Fig. 8A, arrows a and b respectively).

In contrast to the three major sclerotial polypeptides of C. psychromorbidus (LRS131), observed after one-

Table II. A comparison of the major sclerotial polypeptide from cultured and field grown sclerotia in M. borealis.

	Molecular mass (kD)	Relative proportion (% of total)
Cultured	33.9	20
Field Grown	33.9	42

Figure 8. Collage of two-dimensional polyacrylamide gels of polypeptides extracted from mature sclerotia. A. Sclerotial polypeptides of M. borealis (W51). Arrows a and b indicate two major sclerotial polypeptides with pI values of 6.5 and 6.7 respectively. B. Sclerotial polypeptides of C. psychromorbidus (LRS131). Arrows c, d, e and f indicate four major low molecular mass sclerotial polypeptides with pI values ranging from 7.0 to 7.25. Arrow g indicates the 53.7 kD polypeptide with a pI value of approximately 7.0. C. Sclerotial polypeptides of T. idahoensis (W21). Arrows h, i and j indicate three major high molecular mass polypeptides with pI values ranging from 7.25 to 7.5 respectively. Arrow k indicates the low molecular mass polypeptides which have pI values of approximately 7.7. D. Sclerotial polypeptides of T. incarnata (W29). Arrows l and m indicate the two major polypeptides with pI values of 7.5 and 7.6 respectively. Molecular standards were the same as in Figure 2.


Mol.
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30
20
14
pH
Mol.
Mass (kD)
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94
67
43
30
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14
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5.8
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dimensional SDS-PAGE, four polypeptides with pI values of approximately 7.0 to 7.25 (Fig. 8B, arrows c, d, e and f) were observed after two-dimensional electrophoresis. One polypeptide appeared to migrate as a 14.5 kD polypeptide (Fig. 8B, arrow c); two appeared to migrate as 17.0 kD polypeptides (Fig. 8B, arrows d and e); and one appeared to migrate as an 18.2 kD polypeptide (Fig. 8B, arrow f). Two-dimensional gel electrophoresis resolved the major high molecular mass moiety (53.7 kD) of LRS131 as a single polypeptide (Fig. 8B, arrow g) with a pI value of approximately 7.0.

An obvious discrepancy existed between the relative amounts of the major sclerotial polypeptides of T. idahoensis (W21) in the one-dimensional slab gel (Fig. 6C) and the two-dimensional slab gel (Fig. 8C). Under the conditions of one-dimensional SDS-PAGE, the low molecular mass (16.2-21.9 kD) polypeptides predominated (Fig. 6C, lane 4). However, two-dimensional gel electrophoresis indicated that the high molecular mass (72.4 kD) polypeptide predominated but was resolved as three polypeptides ranging in pI values from 7.25 to 7.5 (Fig. 8C, arrows h, i and j). The major low molecular mass polypeptides (16.2-21.9 kD) of W21 were barely visible after the two-dimensional electrophoretic separation and had pI values of approximately 7.7 (Fig. 8C, arrow k).

Although the one-dimensional separation of the sclerotial polypeptides of T. incarnata (W29) indicated the

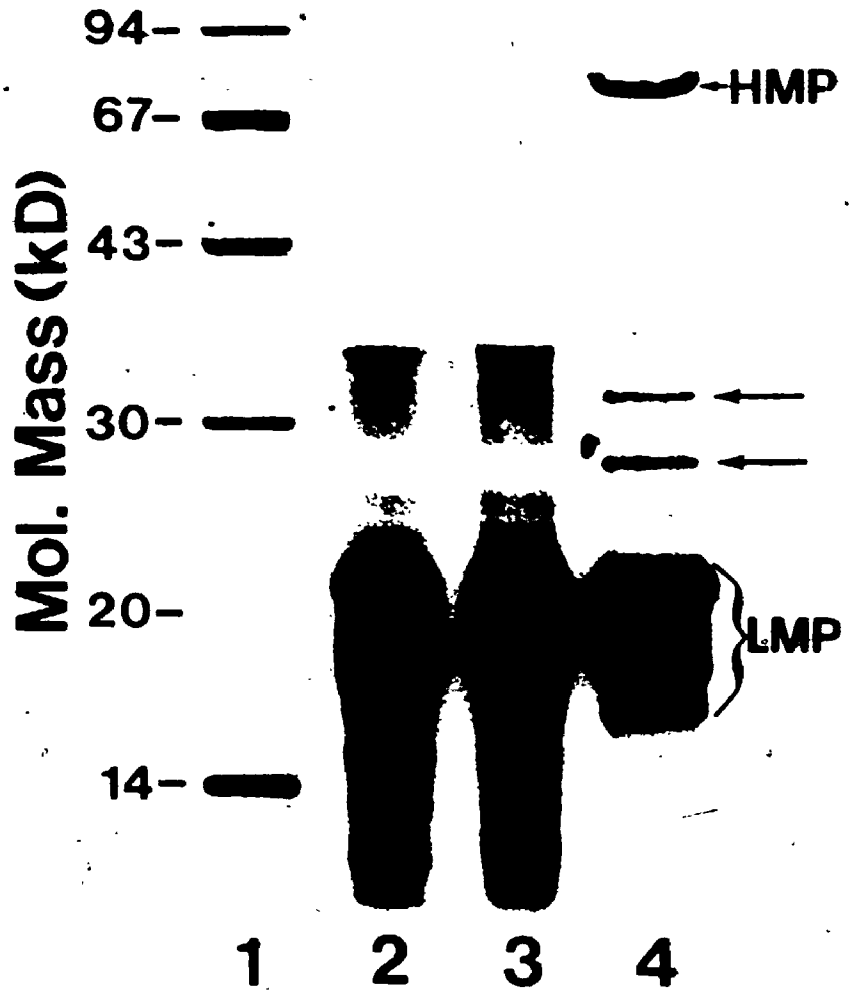
presence of a major (17.0 kD) and minor (18.6 kD) low molecular mass polypeptide, two-dimensional separation indicated the presence of two major polypeptides of approximately equal proportions (Fig. 8D, arrow l and m). The molecular masses of these polypeptides were approximately 17.0 and 18.6 kD with pI values of about 7.5 and 7.6 respectively.

2.3.4 One-dimensional PAGE of sclerotial proteins of W21 and W29 subjected to different denaturing conditions.

One-dimensional PAGE employs SDS, an anionic detergent, as a denaturant whereas, in two-dimensional PAGE, urea is used as the initial denaturant. These compounds denature proteins by different mechanisms (Creighton, 1983). One possible explanation for the discrepancies observed between the one-dimensional and two-dimensional electrophoretic separations of the sclerotial polypeptides of W21 and W29 is the effect of initial protein denaturation with urea on the polypeptide complement observed subsequently by SDS gel electrophoresis.

Electrophoresis of sclerotial protein of W21, which was solubilized in SDS sample buffer alone, showed a predominance of low molecular mass polypeptides (Fig. 9, lane 2, designated LMP's) which constituted 48% of the total sclerotial polypeptide complement (Table III). The major high molecular mass polypeptide (Fig. 9, lane 2, designated HMP) constituted only 0.4% of the total

Figure 9. One-dimensional SDS polyacrylamide gel comparing the effects of urea on the sclerotial polypeptide profile of T. idahoensis (W21). Lane 1, standard proteins; lane 2, sclerotial protein of W21 subjected to SDS solubilization; lane 3, sclerotial protein of W21 subjected to SDS solubilization with subsequent addition to 4.5 M urea; lane 4, sclerotial protein of W21 subjected to urea solubilization with subsequent addition of SDS. HMP designates the major high molecular mass polypeptide (72.4 kD). LMP designates the major low molecular mass polypeptides (16.2 - 21.9 kD). Unmarked arrows indicate the two new prominent polypeptides which appear when sclerotial protein of W21 is initially solubilized in urea. Molecular standards were the same as in Figure 2.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Mol. Mass (kD)
HMP
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Table III. The effect of denaturants on the relative proportions of the major sclerotial polypeptides in T. idahoensis and T. incarnata. Samples were initially solubilized in either SDS buffer, SDS buffer which was subsequently brought to 4.5 M urea or urea buffer which was subsequently brought to 2.3% (w/v) SDS.

Species	Polypeptide moieties	Relative proportion (% of total)		
		SDS only	SDS + 4.5 M urea	Urea + SDS
T. idahoensis	LMP[a]	48	47	56
	HMP[b]	0.4	0.6	5.1
T. incarnata	M_1[c]	36	40	30
	M_2[d]	16	14	20

[a] LMP, low molecular mass polypeptides (16.2-21.9 kD range).

[b] HMP, high molecular mass polypeptide (72.4 kD moiety).

[c] M_1, low molecular mass polypeptide (17.4 kD moiety).

[d] M_2, low molecular mass polypeptide (15.8 kD moiety).

polypeptide complement (Table III). The LMP/HMP ratio was 120. The addition of urea to sclerotial proteins of W21, previously solubilized in normal SDS sample buffer, appeared to have little effect on either the polypeptide profile observed (Fig. 9, lane 3) or the relative amounts of the LMPs and HMP (Table III). In contrast, electrophoresis of the same sclerotial protein preparation of W21, after initial solubilization in urea buffer, showed an altered polypeptide profile (Fig. 9, lane 4) and a tenfold increase in the relative amount of the HMP (Table III) such that the LMP/HMP was reduced to 11. This increase was clearly evident when comparing the absorbance scans (Fig. 10). In addition, two new prominent polypeptides of approximately 33.9 and 28.2 kD became apparent (Fig. 9, lane 4, upper and lower unmarked arrows). There was also a small (8%) increase (Table III) in the relative amount of LMP when denaturation initially occurred in urea.

Electrophoresis of sclerotial protein of T. incarnata (W29), which was solubilized in SDS sample buffer alone, showed a predominance of a major low molecular mass (about 17.4 kD) polypeptide (Fig. 11, lane 2, designated M_1) which comprised 36% of the total sclerotial polypeptide complement (Table III). Another low molecular mass (about 15.8 kD) polypeptide (Fig. 11, lane 2, designated M_2) was also present and constituted 14% of the total sclerotial polypeptide complement (Table III). The M_1/M_2 ratio was 2.3. The addition of urea to sclerotial proteins of W29,

Figure 10. Scans showing the effects of urea on the relative amounts of sclerotial polypeptides of T. idahoensis (W21). 1, scan of sclerotial polypeptides of W21 subjected to SDS solubilization; 2, scan of sclerotial polypeptides of W21 subjected to SDS solubilization with subsequent addition to 4.5 M urea; 3, scan of sclerotial polypeptides of W21 subjected to urea solubilization with the subsequent addition of SDS. Small arrows denote the top and the bottom of the gel. Arrow A indicates the HMP. Arrows B and C indicate the 33.9 and 28.2 kD polypeptides which occurred after initial solubilization in urea. Long arrow indicates the direction of electrophoresis. All scans were performed at a wavelength of 555 nm.

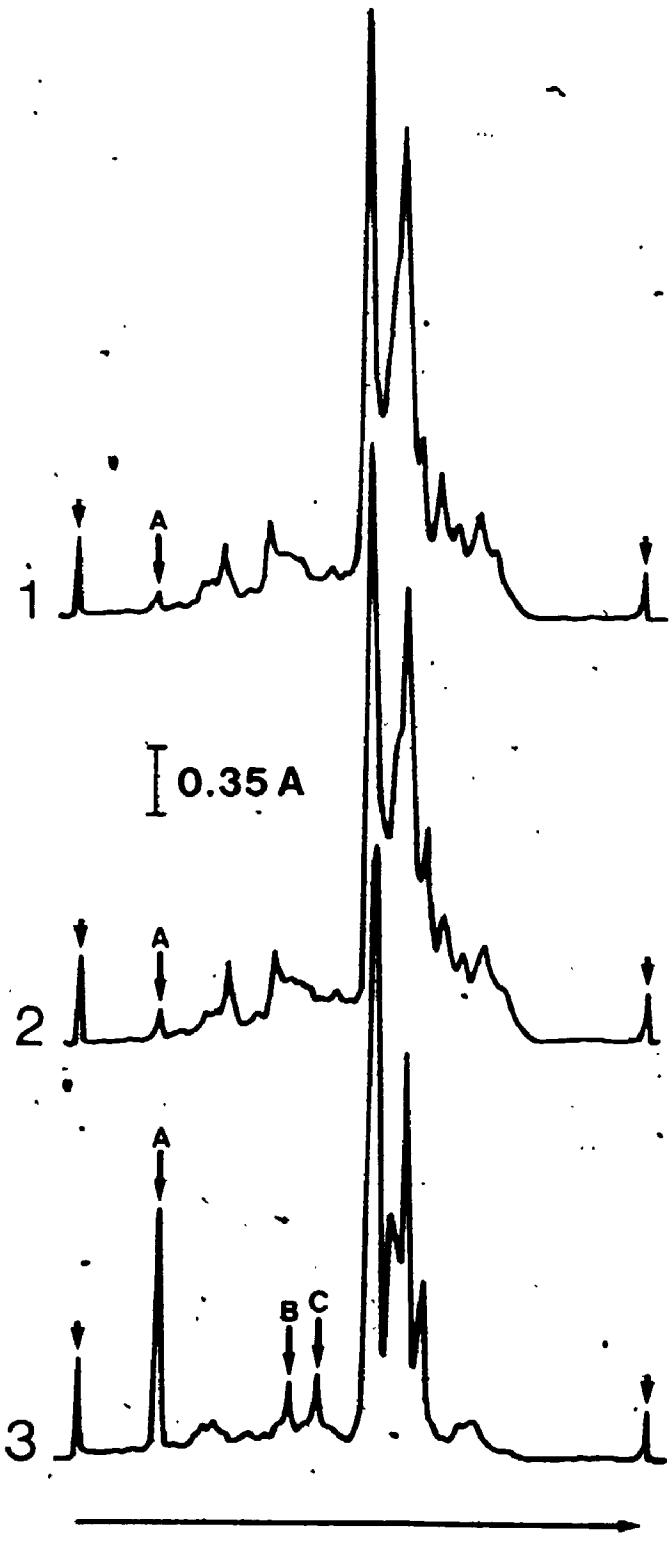
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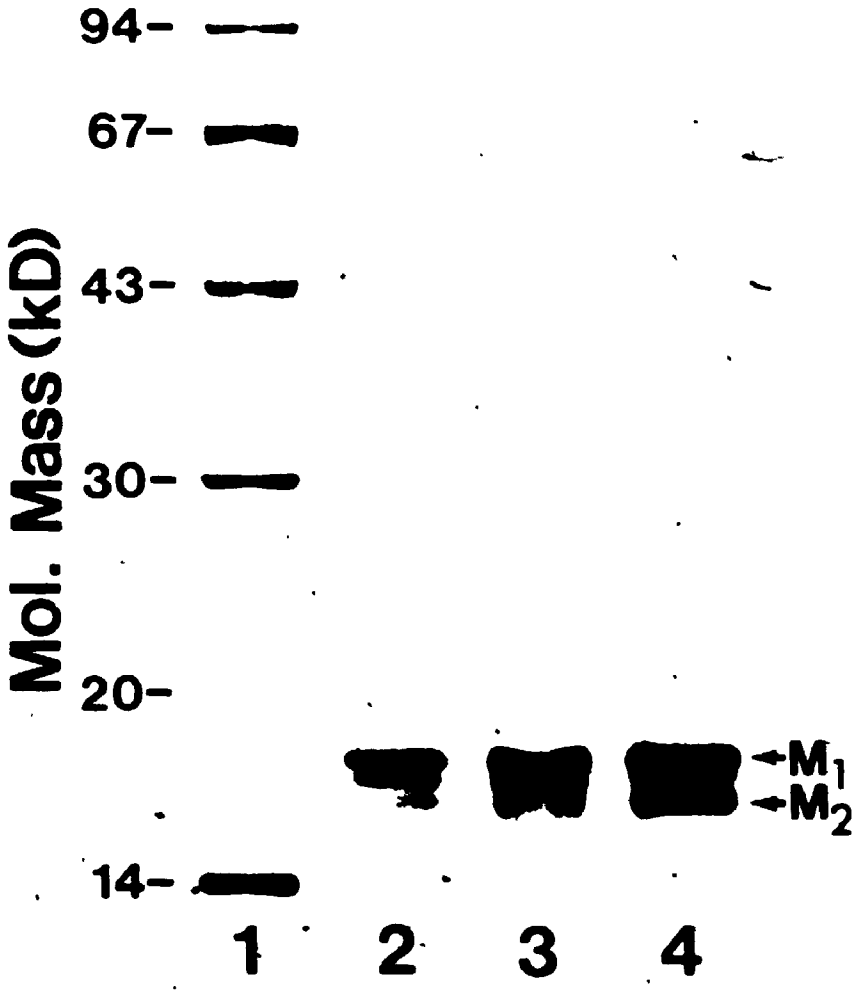
Figure 11. One-dimensional SDS polyacrylamide gel comparing the effects of urea on the sclerotial polypeptide profile of T. incarnata (W29). Lane 1, standard proteins; lane 2, sclerotial protein of W29 subjected to SDS solubilization; lane 3, sclerotial protein of W29 subjected to SDS solubilization with subsequent addition of 4.5 M urea; lane 4, sclerotial protein of W29 subjected to urea solubilization with subsequent addition of SDS. M_1 denotes the major low molecular mass polypeptide of 17.4 kD. M_2 denotes the major low molecular mass polypeptide of 15.8 kD. Molecular standards were the same as in Figure 2.


94-
67-
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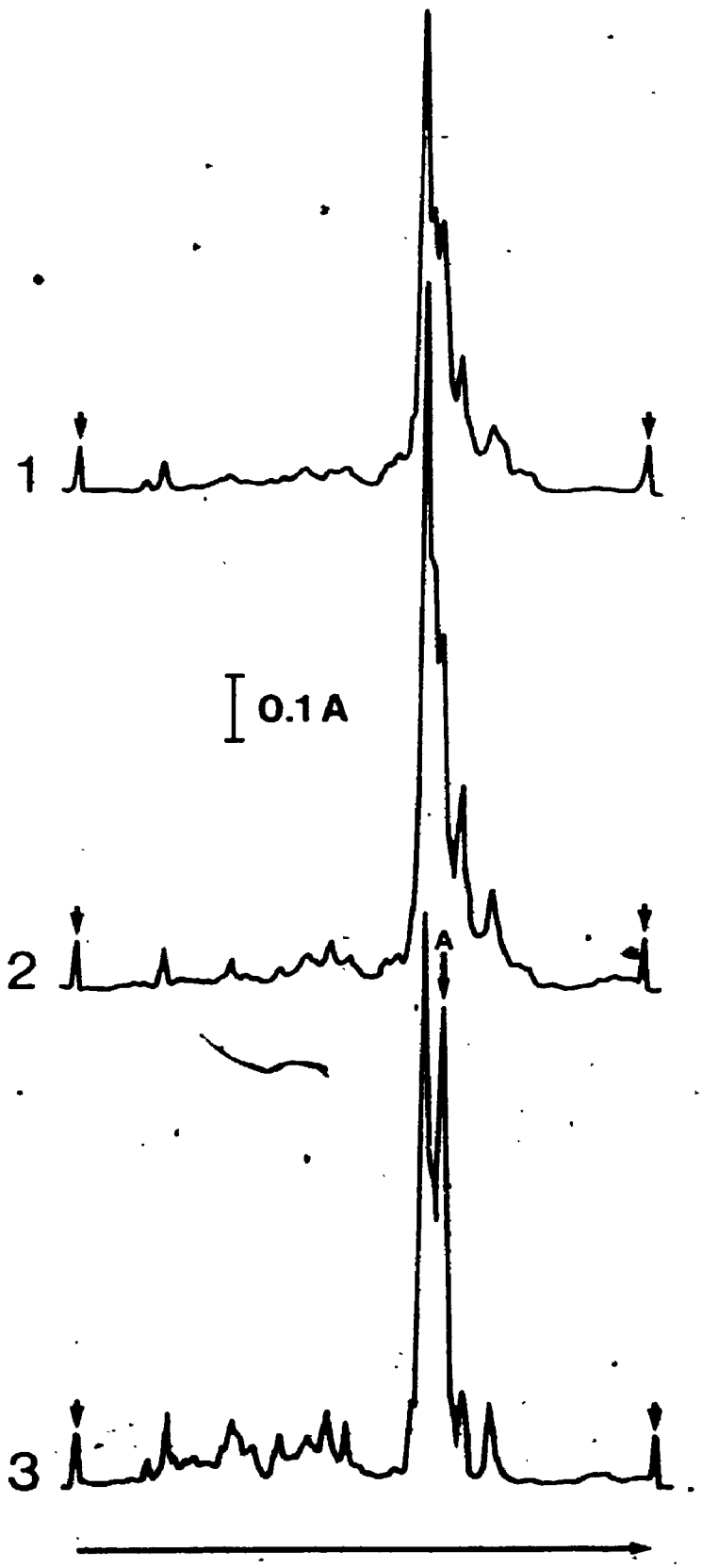
20-
14-
Mol. Mass (kD)
M1
M2
1
2
3
4

which had been previously solubilized in normal SDS sample buffer, appeared to have little effect on either the polypeptide profile observed (Fig. 11, lane 3) or the relative amounts of the M_1 and M_2 polypeptides. However, electrophoresis of the same sclerotial protein preparation of W29 originally solubilized in urea resulted in a similar proportion of M_1 and M_2 (Fig. 11, lane 4). This observation was also evident in the absorbance scans (Fig. 12). Under these conditions, M_1 comprised 30% and M_2 comprised 20% of the total sclerotial polypeptide complement (Table III) and resulted in a M_1/M_2 ratio of 1.5.

2.4 DISCUSSION

This chapter furnishes the first evidence that sclerotia of M. borealis, C. psychromorbidus, T. idahoensis and T. incarnata contain large amounts of specific proteins. The number and molecular weight of the major sclerotial polypeptides when initially denatured in the presence of SDS vary from species to species. Polypeptides of similar molecular weight were generally apparent in the sclerotial initials, an intermediate stage, but were either absent or present in very low quantities in the vegetative hyphae. On the basis of similar molecular mass, it may be suggested that the major polypeptides present in the sclerotial initials and mature sclerotia are related.

Figure 12. Scans showing the effects of urea on the relative amounts of sclerotial polypeptides of T. incarnata (W29). 1, scan of sclerotial polypeptides of W29 subjected to SDS solubilization; 2, scan of sclerotial polypeptides of W29 subjected to SDS solubilization with subsequent addition to 4.5 M urea; 3, scan of sclerotial polypeptides of W29 subjected to urea solubilization with the subsequent addition of SDS. Small arrows denote the top and bottom of the gel. Arrow A indicates M_2. Long arrow indicates the direction of electrophoresis. All scans were performed at a wavelength of 555 nm.


1
0.1 A
2
A
3

Numerous reports of specific gene products associated with development in mesophilic fungi have been published. Nashrallah and Srb (1973, 1977) discovered a major protein associated with perithecial formation in Neurospora crassa. Van Etten et al. (1979) detected a major protein in the spores of Botryodiplodia theobromae which decreased in concentration during spore germination. Also, Champe et al. (1981) have shown that condia and ascospores of Aspergillus nidulans contain a protein which occurs simultaneously with spore formation. Thus, the expression of major gene products in both the pre-sclerotial stage and mature sclerotia of the snow molds may represent a developmentally regulated phenomenon.

Petersen et al. (1982) reported the presence of a single major protein of about 30-33 kD in the sclerotia of three mesophilic Sclerotinia species: S. trifoliorum, S. minor and S. sclerotiorum. It is interesting from a taxonomic viewpoint that Myriosclerotinia borealis, formerly Sclerotinia borealis (Bub and Vleug.), also contains a major polypeptide of approximately the same molecular mass (33.9 kD) as the three Sclerotinia species studied by Petersen et al. (1982).

However, this study has provided several unique insights into the major polypeptides associated with sclerotial development. Firstly, in contrast to the Sclerotinia species, these studies showed that the psychrophiles, C. psychromorbidus, T. idahoensis and T.

incarnata, contained more than one prominent sclerotial polypeptide. The molecular masses of the most prominent polypeptides were significantly lower than those reported by Petersen et al. (1982) and ranged from about 13-22 kD. In addition, the sclerotia of C. psychromorbidus and T. idahoensis showed the presence of major high molecular mass polypeptides of 53.7 and 72.4 kD respectively. Secondly, Petersen et al. (1982) examined the polypeptide composition of mature sclerotia and vegetative hyphae only. However, this study has shown that polypeptides of similar molecular mass to those observed in mature sclerotia were also present, to varying degrees, in sclerotial initials, a developmental form intermediate between vegetative hyphae and mature sclerotia. Thirdly, Petersen et al. (1982) found that the major proteins of Sclerotinia species comprised 31 to 38% of the total sclerotial protein. In this study, it was also found that the major protein of M. borealis, a psychrophile closely related to the Sclerotinia species, comprised approximately 30% of the total sclerotial protein. However, the sclerotia of the other three psychrophilic species examined contained major polypeptides which constituted from 48 to 60% of the total polypeptide complement. Thus, this study has shown that C. psychromorbidus and the two Typhula species exhibit the presence of numerous major sclerotial polypeptides of significantly lower molecular mass and constitute a greater relative proportion of the total polypeptide complement

than that observed for sclerotial-forming Sclerotinia species.

Lastly, two-dimensional electrophoresis of the sclerotial polypeptides of W51 indicated that the major sclerotial polypeptides had pI values which were less acidic than those reported by Russo et al. (1982) for the mesophilic Sclerotinia species. Isoelectric points of 6.5 to 6.7 were reported in this study whereas they report isoelectric points of 5.8 to 6.2. In contrast, the major sclerotial polypeptides of C. psychromorbidus, T. idahoensis and T. incarnata generally exhibited more basic pI values ranging from 7.0 to 7.7.

The presence of a major polypeptide (33.9 kD) in cultured sclerotia of W51 does not appear to be an artifact of culturing conditions because a major polypeptide of similar molecular mass was also present in field grown sclerotia of M. borealis. However, the presence of two other prominent polypeptides in field sclerotia, coupled with the fact that the relative proportion of the major sclerotial polypeptide was 20% higher in the field sclerotia, indicated that there were also subtle differences in the polypeptide complement of cultured versus field sclerotia. This may be partly due to the different physical and/or chemical factors to which the field sclerotia were subjected during development, the age of the sclerotia, or to inherent dissimilarities between different isolates of the same species.

The results presented here indicated some variation in the overall polypeptide profiles of W51 cultured sclerotia as seen in Figure 6A, lane 4 and Figure 7, lane 2. In addition, variation was found in the relative proportion of the W51 major sclerotial polypeptide obtained from cultured sclerotia. In Figure 6A, lane 4, the major sclerotial polypeptide constituted 30% of the total sclerotial polypeptide complement (Table I), whereas in Figure 2, lane 2, it comprised only 20% of the total sclerotial polypeptide complement (Table II). This may be due to variation in the precise age of the mature sclerotia at the time of extraction.

Discrepancies were found between the relative proportions of the major sclerotial polypeptides observed in one versus two-dimensional gel electrophoresis for the two Typhula species (Figs. 6C and D; Figs. 8C and D). It was concluded that the discrepancies observed in the two-dimensional gels were not a result of protein failing to enter the IEF gel. It was therefore hypothesized that these discrepancies were at least partly due to the effect of the urea buffer employed for isoelectric focussing on subsequent protein denaturation. The results of Fig. 9 indicate that initial solubilization with urea resulted in a tenfold increase in the amount of the high molecular weight sclerotial polypeptide observed in T. idahoensis. In addition, initial solubilization in the presence of urea also resulted in significant changes in the total

polypeptide complement observed (Fig. 9, arrows). Some polypeptides became more apparent whereas others became less apparent when extracts were initially solubilized in urea buffer. Similarly, the results of Fig. 10 indicate that initial solubilization with urea resulted in a substantial increase in the M_2 sclerotial polypeptide and a corresponding decrease in the M_1/M_2 ratio for T. incarnata. However, these urea effects cannot totally account for the discrepancies in one and two-dimensional gels. The isoelectric focussing process itself may exacerbate these urea effects. In contrast, sclerotial polypeptides of M. borealis and C. psychromorbidus appeared to be much less sensitive to the effects of different denaturants since their polypeptide profiles were consistent when comparing one and two-dimensional gels. That the sclerotial polypeptides of T. idahoensis and T. incarnata should show greater sensitivity to the type of denaturant used during solubilization indicates that the biochemical structure of the sclerotial protein from these species may be different. These results support the urea hypothesis and thus caution should be exercised when comparing one-dimensional SDS gels with two-dimensional gels.

Russo et al. (1982) have postulated that the major sclerotial protein of Sclerotinia sclerotiorum may function as a storage protein. More recently, Russo and Van Etten (1985) demonstrated that this protein accumulated in organelles which morphologically resembled protein bodies

in the seeds of plants (Lott, 1980). This evidence, plus data showing decreased levels of the major sclerotial protein in sclerotia after carpogenic germination (Russo et al., 1982), strongly suggest a storage role for these proteins. In this chapter, it has been shown that sclerotia of M. borealis, C. psychromorbidus, T. idahoensis and T. incarnata, like seeds of plants, accumulate major proteins. In Chapter 4 of this thesis, it will also be shown that the major sclerotial polypeptides in the sclerotia of the psychrophilic fungi are sequestered in protein bodies. Thus, it is not unreasonable to propose a storage protein role for the major solerotial polypeptides of the snow molds because sclerotia, like the seeds of plants, represent important perennating structures.

CHAPTER 3

IMMUNOLOGICAL RELATEDNESS OF THE MAJOR SCLEROTIAL POLYPEPTIDES

3.1 INTRODUCTION

In the previous chapter, it was demonstrated for the first time that Myriosclerotinia borealis (W51), Coprinus psychromorbidus (LRS131), Typhula idahoensis (W21) and Typhula incarnata (W29) produced major polypeptides as a function of sclerotial development. This chapter describes the purification of the major sclerotial polypeptides from psychrophilic fungi and the production of polyclonal antibodies to these purified sclerotial proteins. The antibodies were used to determine the immunological relatedness of sclerotial polypeptides of varying molecular mass from psychrophilic and mesophilic species using the Ouchterlony immunodiffusion technique as well as Western blots.

3.2 MATERIALS AND METHODS

3.2.1 Protein extraction.

Cultures were grown as previously described (Chapter 2) to produce mature sclerotia of each species. Mature sclerotia were collected from the surface of the agar,

pulverized in a mortar with a pestle at 0°C and then extracted in a buffer containing proteolytic inhibitors as previously described (Chapter 2). Protein concentration was determined using the Bio-Rad protein assay according to Bradford (1976). Sclerotial protein samples were solubilized in a minimal amount of a SDS sample buffer previously described (Chapter 2). Samples were stored at -70°C until required.

3.2.2 Purification of the major sclerotial polypeptides.

Protein purification of the major sclerotial polypeptides was carried out according to the methods described by Baszczynski (1986) and Bhown et al. (1980) with several modifications. Samples consisting of 1 mg of extracted sclerotial protein in 300 µl of sample buffer were loaded into large wells (10.5 cm x 1.5 mm x 2.5 cm) of a one-dimensional SDS polyacrylamide gradient gel (12-18%) overlayed with a 5% stacking gel. In addition, a 4 µl sample of standard proteins from a low molecular weight calibration kit (Pharmacia Fine Chemicals) was loaded onto a single outer well to assist in localizing the major sclerotial polypeptide bands in the gel on the basis of molecular weight. The running buffer consisted of 0.05 M Tris buffer, 0.4 M glycine, 0.1% (w/v) SDS and 0.2 M EDTA (pH 8.3). The sclerotial polypeptides were electrophoresed in a Bio-Rad Protean dual vertical slab gel electrophoresis cell at 25 mA per gel for approximately 5 hours in a manner

similar to Laemmli (1970).

After electrophoresis, a small vertical slice of the gel, which included the standard protein lane plus a narrow strip of the sclerotial polypeptide lane, was excised and stained for 5 minutes in a solution of 0.1% (w/v) Coomassie Brilliant Blue in 40% (v/v) methanol and 10% (v/v) acetic acid and then destained to visualize the major sclerotial polypeptides. Sections from the unstained slab gels corresponding to regions containing the major sclerotial polypeptides were cut out carefully. These included the 31-33 kD major sclerotial polypeptide of W51; the three major sclerotial polypeptides of LRS131 in the 12.9-14.5 kD range; the five major sclerotial polypeptides of W21 in the 16.2-21.9 kD range; the major high molecular mass (72.4 kD) sclerotial polypeptide of W21; and collectively, the major (17 kD) and minor (18.6 kD) sclerotial polypeptides of W29. The sections containing the major sclerotial polypeptides were chopped into small pieces (approximately 1 x 1 x 1.5 mm) and transferred into ISCO Model 1750 Sample Concentrator cups. The gel pieces were placed in the large chamber and the small chamber was filled with 200 µl of a 0.01 M Tris-acetate buffer (pH 8.6) containing 10% sucrose. The remainder of the cup was filled carefully with 10 ml of 0.01 M Tris-acetate-buffer (pH 8.6) without sucrose.

The cups were placed across a partition in a buffer tank such that each end of the cup was immersed in 0.04 M Tris-acetate buffer (pH 8.6). The polypeptides were then

electroeluted from the gel pieces for 4-5 hours at 2 to 3 watts per cup. After electroelution, the concentrated polypeptide solutions (200 µl) were then removed from the small chambers. Protein concentration was determined using the turbidimetric method of Comings and Tack (1972). Purity was checked by re-electrophoresis.

3.2.3 Antibody production.

White, female, New Zealand rabbits were obtained and allowed to acclimatize to their new environment for 2-3 weeks before being used for immunological studies. Pre-immune serum was prepared by collecting a blood sample before injecting the rabbits with antigen. To prepare pre-immune serum, a small incision was made across the marginal ear vein at the base of the ear. Approximately 40 ml of whole blood was collected in a 50 ml plastic centrifuge tube. The blood was incubated at 37°C for 30 minutes and then at 4°C for 24 hours to promote clotting. The clotted blood sample was then centrifuged at 1500 xg for 20 minutes. The amber serum was collected and transferred to 15 ml conical tubes and centrifuged at 1500 xg for 10 minutes to remove residual red blood cells. The sera were placed at 56°C for 30 minutes to inactivate complement and then stored at -70°C until required.

Rabbits were injected intramuscularly in the right hind leg with the prepared antigens, 400 µg in complete Freund's adjuvant (1:1). One rabbit was injected with the 31-33 kD

major sclerotial polypeptide of W51, one with the 12.9-14.5 kD major sclerotial polypeptides of LRS131, one with the 16.2-21.9 kD major sclerotial polypeptides of W21, and one with the major (17 kD) and minor (18.6 kD) sclerotial polypeptides of W29. Two weeks later, rabbits were boosted with antigens in incomplete Freund's adjuvant (1:1) via an intramuscular injection in the left hind leg. Rabbits were bled 2 weeks later.

To prepare the antiserum, a small incision was made across the marginal ear vein upstream from where the incision had been made to collect blood for pre-immune serum. Approximately 40 ml of whole blood was collected from each rabbit. The whole blood was allowed to clot and the antisera were prepared as described previously for pre-immune sera. The immunoglobulins were precipitated from crude antisera with 27% (w/v) ammonium sulphate, stirred slowly for 2 hours at room temperature and then centrifuged for 30 minutes at 15,000 xg. The pellets were resuspended in about 4 or 5 ml of buffer containing 15 mM potassium phosphate and 50 mM NaCl (pH 7.0). Samples were then dialysed against the same buffer for 16 hours at 4°C. The buffer was changed four times during dialysis. The dialysates were subsequently stored in 200 µl aliquots at -70°C.

3.2.4 Ouchterlony immunodiffusion.

Ouchterlony plates were prepared by pouring 3 ml of

melted 1.2% Noble agar in 50 mM sodium barbituate buffer (pH 8.6) onto clean microscope slides. Wells were cut out using a standard gel hole puncher. The centre well in each case was filled with 20 µl of one of the concentrated antisera. The six surrounding wells were filled with samples of crude or purified sclerotial polypeptides obtained from the various fungal species. Plates were incubated overnight in sealed petri dishes.

In all experiments, pre-immune serum was run concurrently against the major sclerotial polypeptides to test the specificity of the various antisera for their respective antigens.

3.2.5 Western blot protocol.

Crude sclerotial protein extracts from each snow mold species were prepared as previously described at the beginning of the Methods section in this chapter. Samples consisting of 100 ng of crude sclerotial protein from each species were loaded into separate wells of four independent one-dimensional SDS polyacrylamide gradient gels (12-20%) overlayed with a 5% stacking gel. In addition, a 4 µl sample of standard proteins from a low molecular weight calibration kit (Pharmacia Fine Chemicals) was loaded onto a single outer well to assist in molecular mass determination. The crude sclerotial polypeptides were electrophoresed for 3 hours at 10°C at a constant current of 15 mA per gel using a Mighty Small II Slab Gel

Electrophoresis Unit SE250 (Hoefer Scientific Instruments) equipped with a water-jacketed chamber with running tap water as coolant. Electrophoresis was continued until the bromophenol blue dye ran off the bottom of the resolving gels.

At the end of the electrophoretic run, the slab gels were removed from the plates and prepared for electro-blotting as outlined by Tsang et al. (1983). A sponge pad was pre-soaked in a transfer buffer consisting of 25 mM Tris (pH 8.35), 192 mM glycine in 20% methanol and placed on a plastic holder. A piece of wet Whatman No. 1 filter paper soaked in transfer buffer was placed on the sponge and the slab gel was carefully lowered onto the filter paper avoiding trapped air bubbles. A sheet of pre-soaked nitrocellulose membrane (Schleicher and Schuell, Inc.), or alternatively, a sheet of Immobilon membrane (Millipore) was placed on the gel surface, again avoiding trapped air bubbles. Another piece of pre-soaked Whatman No. 1 filter paper was placed on top of the membrane and then another pre-soaked sponge was placed on top of the filter paper. The sandwich was completed by placing a second plastic holder on top which clamped into the first plastic holder. The entire sandwich was placed in the TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments) such that the membrane was anodal to the gel. Four mini-gels could be electro-blotted at a given time using this apparatus. The cell was filled with transfer buffer and

the sclerotial proteins were electro-transferred onto the membranes for 16 hours at 60 volts (constant voltage) using a Bio-Rad Model 25012.5 constant voltage power supply. In addition, a cooling coil was inserted into the cell to keep the buffer cool during electro-transfer.

3.2.6 Membrane processing using enzyme-linked immunoassay.

After electrophoretic transfer, the membranes were carefully removed from the gels. The section onto which standard proteins had been transferred from the gels was carefully excised, stained with 1% (w/v) Amido black in 7% (v/v) methanol; 7% (v/v) acetic acid for 10 seconds and destained in 40% (v/v) methanol; 7.5% (v/v) acetic acid until the background cleared. These strips served as reference lanes for molecular mass determinations.

The rest of the nitrocellulose (or Immobilon) membranes, retaining the total sclerotial polypeptide profiles of all four species, were washed four times for 15 minutes each with phosphate-buffered saline consisting of 0.01 M Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl (pH 7.2) and 0.3% (v/v) Tween 20 (PBS-Tween). Tween 20 blocks most of the remaining sites on the membrane and helps to minimize non-specific binding of the primary antibodies to the membranes. Following this step, the four membranes were transferred to separate square plastic petri dishes. Each dish contained a different primary antibody solution. For each species, this consisted of a 1:2500 dilution of

antiserum raised against its major sclerotial polypeptides in PBS-Tween. The membranes were incubated in this solution for 2 hours with gentle agitation. They were then removed from the primary antibody solution, washed three times in PBS-Tween for 10 minutes and transferred to the secondary antibody solution consisting of a 1:3000 dilution of goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate [Bio-Can] in PBS-Tween. The membranes were incubated in this solution for 1 hour with gentle agitation. The membranes were then removed and washed twice in PBS-Tween for 10 minutes, followed by a final rinse in Tris-buffered saline (TBS) containing 20 mM Tris-HCl (pH 7.4) and 500 mM NaCl for 10 minutes. This removes the Tween 20 prior to colour development.

The HRP colour development solution was prepared by combining 10 ml of TBS, 50 µl of 0.5% (w/v) o-dianisidine dihydrochloride in 100% (v/v) methanol and 3 µl of 30% (v/v) H_2O_2. This solution was prepared immediately prior to use. The membranes were immersed in the HRP colour development solution and the colour reaction was monitored for the next 30 minutes. The reaction was stopped when brown bands were visible and background staining was low or absent. The colour reaction was terminated by gently rinsing the membranes in two 10 minute changes of distilled water. The membranes were then placed in fresh TBS and stored at 5°C until photographed. The membranes were photographed while wet using 35 mm Technical Pan 2415

film (Kodak).

3.3 RESULTS

3.3.1 Purity of major sclerotial polypeptides.

SDS-PAGE of sclerotial polypeptides after electro-elution indicated a purity of 91-98% for the major polypeptides from C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) (Fig. 13). Samples of purified sclerotial polypeptides of W21 exhibited the presence of anomalous polypeptides in a molecular mass range of 36-40 kD (Fig. 13, parenthesis). However, addition of increasing concentrations of the reducing agent, ß-mercaptoethanol, prior to SDS-PAGE resulted in a marked decrease in the proportion of the 36-40 kD polypeptides and a concomitant increase in the major sclerotial polypeptides (16.2-21.9 kD) of W21 (Fig. 14 and Table IV).

The electroeluted sample from W51 exhibited a purity of about 65% based on SDS-PAGE (Fig. 13) which constituted approximately a 2.6-fold increase in purity.

3.3.2 Ouchterlony immunodiffusion.

The results of the Ouchterlony immunodiffusion are illustrated in Fig. 15 (A, B, C and D) and the resultant cross-reactivities are summarized in Table V. All antisera (Fig. 15, wells w, x, y and z) reacted positively against

Figure 13. One-dimensional SDS polyacrylamide gel showing purification of the major sclerotial polypeptides. W51, M. borealis; LRS131, C. psychromorbidus; W21, T. idahoensis and W29, T. incarnata. (C) crude sclerotial polypeptide extracts; (P) purified major sclerotial polypeptides. Parentheses indicate anomalous polypeptides found in purified sclerotial polypeptides of T. idahoensis (W21). Left lane, standard proteins. Molecular standards were: phosphorylase b, 94 kD; bovine serum albumin, 67 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20 kD and α-lactalbumin, 14 kD.

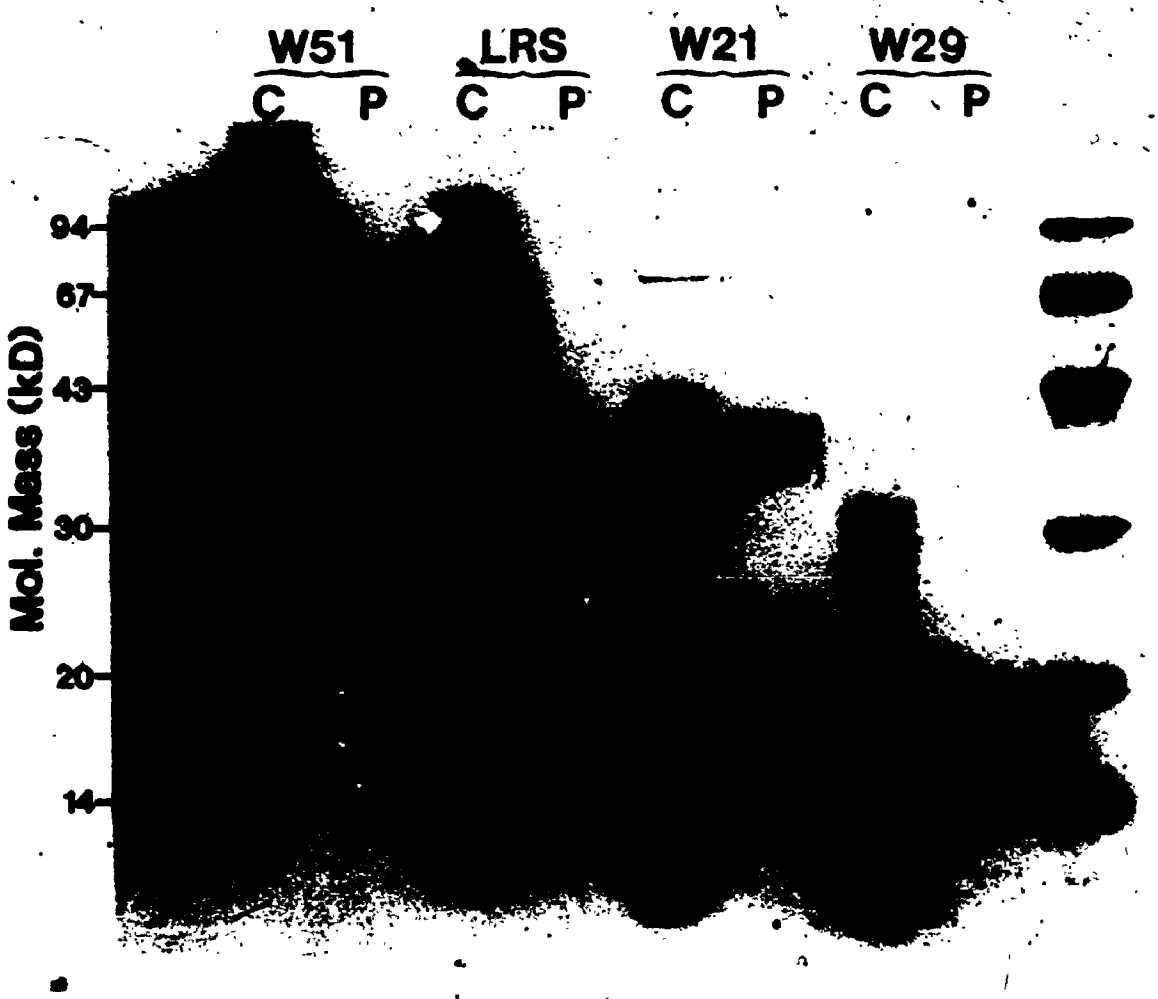

W51
C P
LRS
C P
W21
C P
W29
C P
Mol. Mass (kD)
94
67
43
30
20
14

Figure 14. One-dimensional polyacrylamide gel electrophoresis showing the reduction of aggregates in purified sclerotial polypeptides of T. idahoensis (W21). Lanes 1 and 7, molecular standards; lane 2, crude sclerotial polypeptides of T. idahoensis (W21); lanes 3 through 6, purified low molecular mass sclerotial polypeptides of T. idahoensis (W21) solubilised in the presence of 0, 71, 143 and 285 mM β-mercaptoethanol respectively.


2
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
MicroD

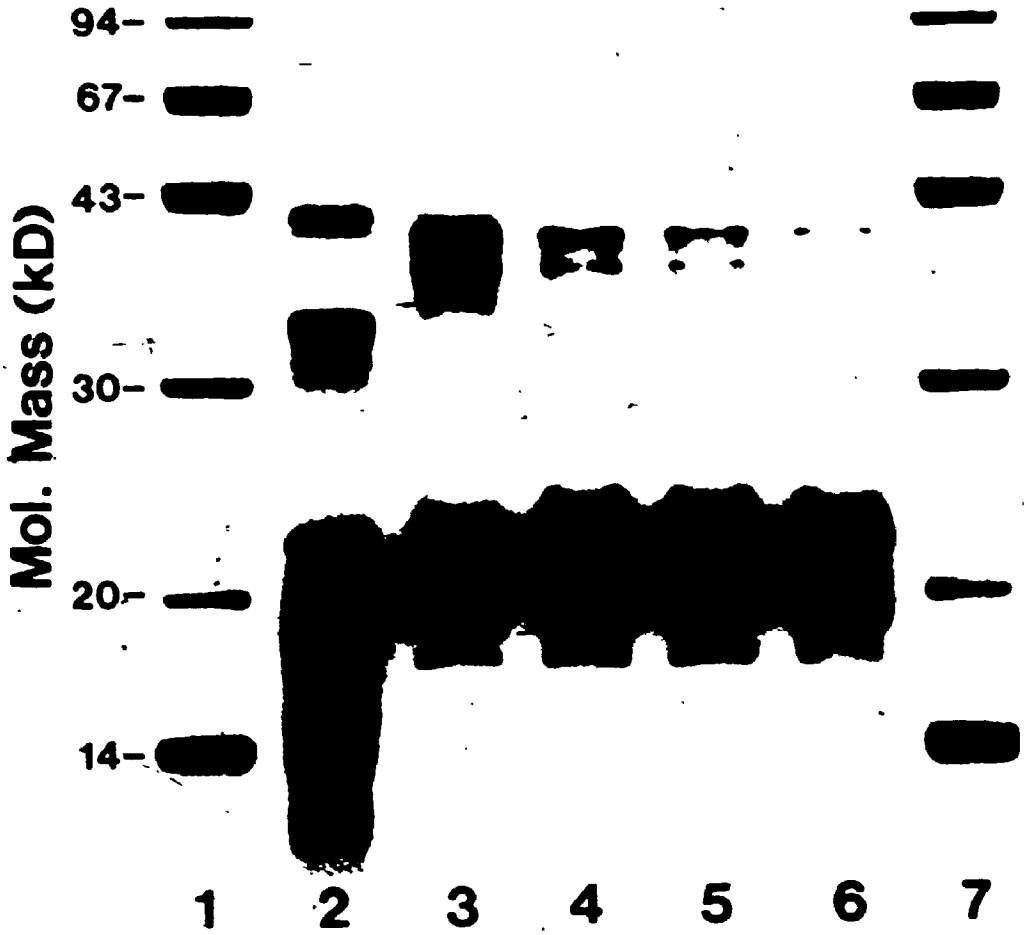

94-
67-
43-
30-
20-
14-
Mol. Mass (kD)
1
2
3
4
5
6
7

Table IV. The effect of increasing concentrations of β-mercaptoethanol on the amount of the 36-40 kD polypeptides observed in purified sclerotial protein of T. idahoensis (W21).

β-mercaptoethanol concentration (mM)	Low molecular mass (16.2-21.9 kD) polypeptides (%)	Anomalous 36-40 kD polypeptides (%)
0.0	57	36
71	86	14
143	87	10
285	95	5

Figure 15. Ouchterlony immunodiffusion analysis of major sclerotial polypeptides.

(A) Centre well (W) contained antiserum to purified M. borealis (W51) sclerotial polypeptides. Well 1, purified sclerotial polypeptides from M. borealis (W51); well 2, pre-immune serum; well 3, buffer; well 4, purified sclerotial polypeptides from T. incarnata (W29); well 5, purified sclerotial polypeptides from T. idahoensis (W21); well 6, purified sclerotial polypeptides from C. psychromorbidus (LRS131).

(B) Centre well (X) contained antiserum to purified C. psychromorbidus (LRS131) sclerotial polypeptides. Well 1, purified sclerotial polypeptides from C. psychromorbidus (LRS131); well 2, pre-immune serum; well 3, buffer; well 4, purified sclerotial polypeptides from T. incarnata (W29); well 5, purified sclerotial polypeptides from T. idahoensis (W21); well 6, purified sclerotial polypeptides of M. borealis (W51).

Cont'd....

Figure 15 cont'd....

(C) Centre well (Y) contained antiserum to purified T. idahoensis (W21) sclerotial polypeptides. Well 1, purified sclerotial polypeptides from T. idahoensis (W21); well 2, pre-immune serum; well 3, buffer; well 4, purified sclerotial polypeptides of T. incarnata (W29); well 5, purified sclerotial polypeptides of C. psychromorbidus (LRS131); well 6, purified sclerotial polypeptides of M. borealis (W51).

(D) Centre well (Z) contained antiserum to purified T. incarnata (W29) sclerotial polypeptides. Well 1, purified sclerotial polypeptides of T. incarnata (W29); well 2, pre-immune serum; well 3, buffer; well 4, purified sclerotial polypeptides of T. idahoensis (W21); well 5, purified sclerotial polypeptides of C. psychromorbidus (LRS131); well 6, purified sclerotial polypeptides of M. borealis (W51). Reactions are summarized in Table 5.

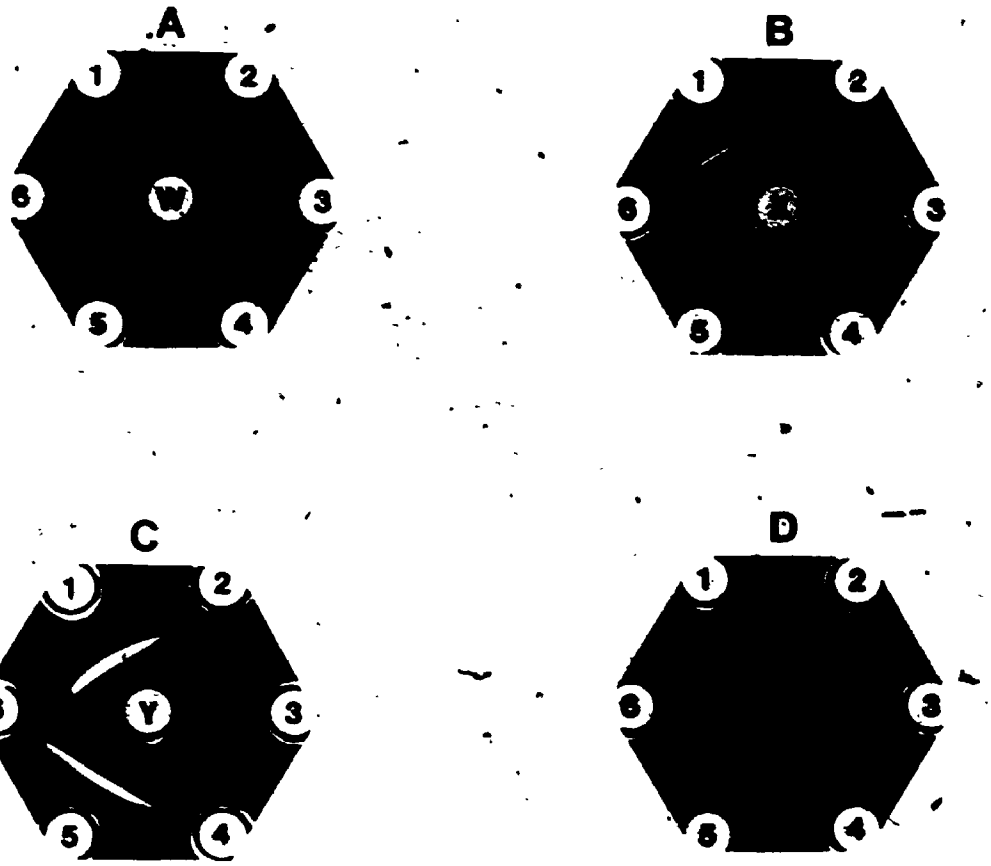

A
1
2
3
4
5
6
W
B
1
2
3
4
5
6
C
1
2
3
4
5
6
Y
D
1
2
3
4
5
6

Table V. Summary of Ouchterlony immunodiffusion reactions.

Antisera	Antigens: Purified Sclerotial Polypeptides			
	W51[a]	LRS131[b]	W21[c]	W29[d]
Anti W51	++	+	+	-
Anti LRS131	-	+++	-	-
Anti W21	-	+++	+++	-
Anti W29	-	w	w	+++

[a] M. borealis

[b] C. psychromorbidus

[c] T. idahoensis

[d] T. incarnata

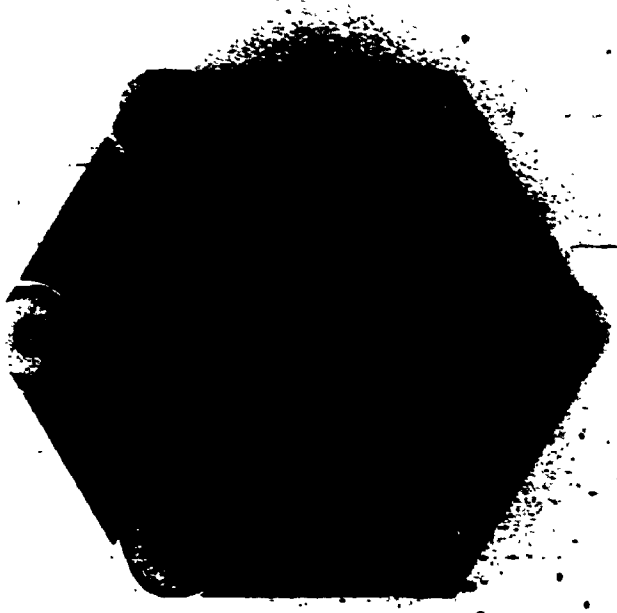
Reactions: +++, very strong; ++, strong; +, moderate; w, weak; -, nil.

their respective antigens (well 1 in each of Figs. 15A, B, C and D). Precipitin arcs were not observed between antisera to purified sclerotial polypeptides and their respective pre-immune sera, nor between antisera and buffer alone.

Antiserum against the purified sclerotial polypeptides of C. psychromorbidus (LRS131) (anti LRS131) appeared to exhibit the greatest specificity. It reacted only against LRS131 sclerotial polypeptides and not against sclerotial polypeptides from the other three psychrophilic species (Fig. 15B; Table V). However, anti LRS131 reacted against crude sclerotial protein extracts of the mesophilic species, Sclerotium rolfsii (Fig. 16, well 6), but not against crude sclerotial extracts of the mesophile, Sclerotinia sclerotiorum (Fig. 16, well 5). The presence of a spur (Fig. 16) indicated that the major polypeptides of C. psychromorbidus (LRS131) were antigenically similar, but not identical to the polypeptides present in sclerotial extracts of S. rolfsii.

Antiserum against the purified sclerotial polypeptides of M. borealis (W51) and T. incarnata (W29) (anti W51 and anti W29 respectively) exhibited the least specificity of the psychrophilic species tested (Figs. 15A and D; Table V). Anti W51 reacted positively to W21 polypeptides (Fig. 15A, well 5) and weakly to LRS131 (Fig. 15A, well 6). This weak reaction could be intensified by increasing the amount of LRS131 protein employed. However, anti W51 did not

Figure 16. Ouchterlony immunodiffusion analysis showing cross-reactivity of antiserum raised to purified sclerotial polypeptides of the psychrophile, C. psychromorbidus (LRS131) with sclerotial proteins of a mesophilic fungus. Centre well (A) contained antiserum to purified C. psychromorbidus (LRS131) sclerotial polypeptides. Well 1, crude C. psychromorbidus (LRS131) sclerotial protein; well 2, pre-immune serum; well 3, buffer; well 4, buffer; well 5, crude S. sclerotiorum sclerotial protein; well 6, crude S. rolfsii sclerotial protein.



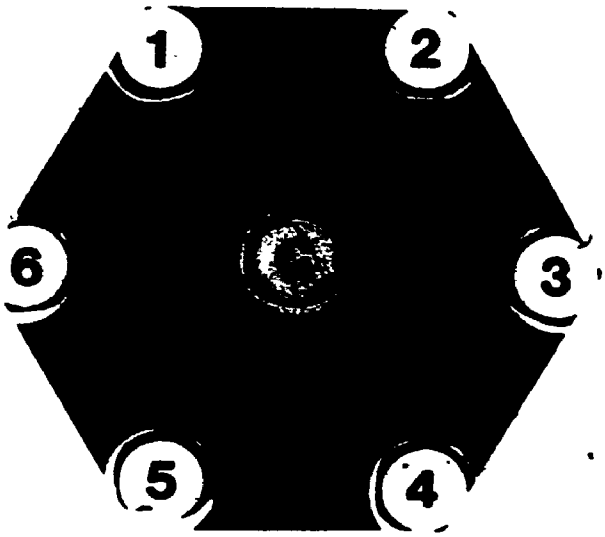
react against W29 polypeptides (Fig. 15A, well 4). Anti W29 (Fig. 15D) reacted positively against both LRS131 (well 5) and W21 polypeptides (well 4), but not against W51 polypeptides (well 6).

Antiserum against the purified sclerotial polypeptides of T. idahoensis (W21) (anti W21) exhibited intermediate specificity (Fig. 15C). It exhibited a very strong reaction with LRS131 polypeptides (Fig. 15C, well 5), but no observable cross-reactivity with either W29 (well 4) or W51 polypeptides (well 6). The purified 72.4 kD polypeptide of mature sclerotia of W21 reacted positively to anti W21 (Fig. 17, well 2). Since no reaction spur was obvious, this 72.4 kD polypeptide appeared to be very similar immunologically to one or more of the major low molecular mass polypeptides (Fig. 17, well 1) observed in sclerotial extracts of this psychrophilic species.

As shown in Chapter 2, field grown sclerotia of M. borealis exhibited a major polypeptide of similar molecular mass (33.9 kD) to that observed for cultured sclerotia of M. borealis. Crude protein extracts of the field grown sclerotia (Fig. 18, well 2) reacted positively to anti W51 and did not exhibit the presence of a spur (Fig. 18). This provided evidence that the major sclerotial polypeptides of field and cultured sclerotia of M. borealis were not only of the same molecular mass but were immunologically similar.

Lastly, it was shown that crude sclerotial protein

Figure 17. Ouchterlony immunodiffusion analysis showing cross-reactivity of antiserum raised to purified low molecular mass (16.2-21.9 kD) sclerotial polypeptides of T. idahoensis (W21) with the purified high molecular mass (72.4 kD) sclerotial polypeptide of T. idahoensis (W21). Centre well (C) contained antiserum to purified low molecular mass (16.2-21.9 kD) sclerotial polypeptides. Well 1, purified low molecular mass (16.2-21.9 kD) sclerotial polypeptides of T. idahoensis (W21); well 2, purified high molecular mass (72.4 kD) sclerotial polypeptide of T. idahoensis (W21); well 3, pre-immune serum; well 4, buffer; well 5, buffer; well 6, buffer.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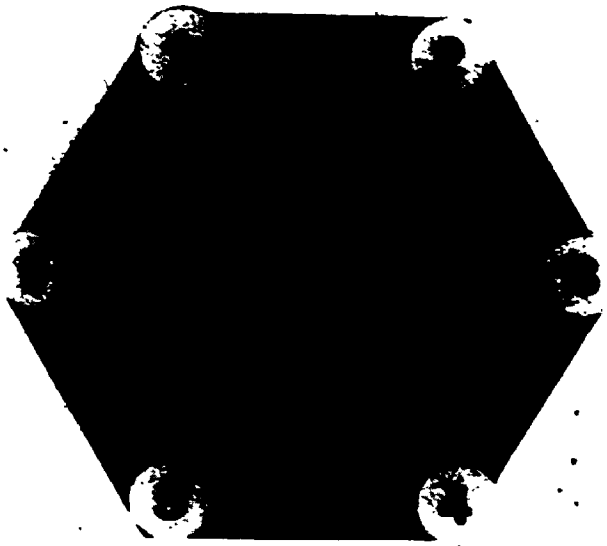
3
4
5
6

Figure 18. Ouchterlony immunodiffusion analysis showing reactivities of antiserum raised to the purified polypeptides isolated from cultured sclerotia of M. borealis (W51) with crude proteins of cultured and field sclerotia of M. borealis (W51). Centre well (A) contained antiserum to the purified M. borealis (W51) sclerotial polypeptide. Well 1, crude protein of M. borealis (W51) from cultured sclerotia; well 2, crude protein of M. borealis (W51) from field sclerotia; well 3, pre-immune serum; well 4, buffer; well 5, buffer; well 6, pre-immune serum.



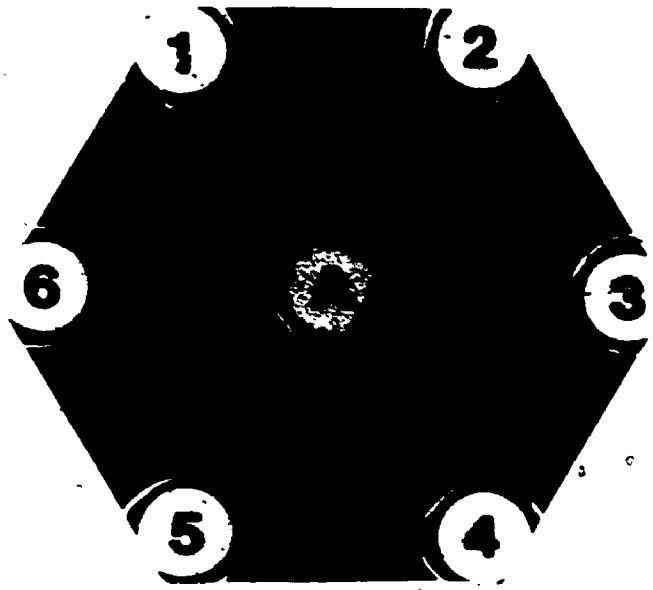
extracts of M. borealis (W51) subjected to denaturation by SDS reacted positively with anti W51 within a 24 hour incubation period (Fig. 19, well 3). However, crude sclerotial protein of M. borealis (W51) extracted under non-denaturing conditions showed no reaction against anti W51 within 24 hours, but required at least 48 hours to produce a positive response. These results indicate that the antigenic site(s) of the major sclerotial proteins of W51 extracted under non-denaturing conditions probably were less accessible to antibody binding.

3.3.3 Western blot analysis.

Antiserum raised against the major sclerotial polypeptide of M. borealis (W51) reacted positively with the major sclerotial polypeptide of M. borealis (W51) (Fig. 20A, lane 1). This was the only sclerotial polypeptide of M. borealis (W51) which reacted positively with the antiserum. A weak cross-reaction was observed against two of the major (12.9-14.5 kD) sclerotial polypeptide bands of C. psychromorbidus (Fig. 20A, lane 2). Cross-reactions were also observed with two of the major sclerotial polypeptides of T. idahoensis in the 16.2-21.9 kD range (Fig. 20A, lane 3). No cross-reaction was observed with any of the sclerotial polypeptides of T. incarnata (W29) (Fig. 20A, lane 4). These results were totally consistent with the Ouchterlony results.

Antiserum raised against the major sclerotial

Figure 19. Ouchterlony immunodiffusion analysis showing reactivities of antiserum raised to the purified sclerotial polypeptide of M. borealis (W51) with denatured and non-denatured sclerotial proteins of M. borealis (W51) over a 24 hour period. Center well (A) contained antiserum to the purified M. borealis (W51) sclerotial polypeptide. Well 1, crude non-denatured sclerotial protein of M. borealis (W51); well 2, pre-immune serum; well 3, crude SDS denatured protein of M. borealis (W51); wells 4 to 6, buffer.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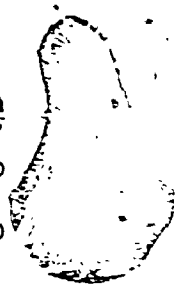
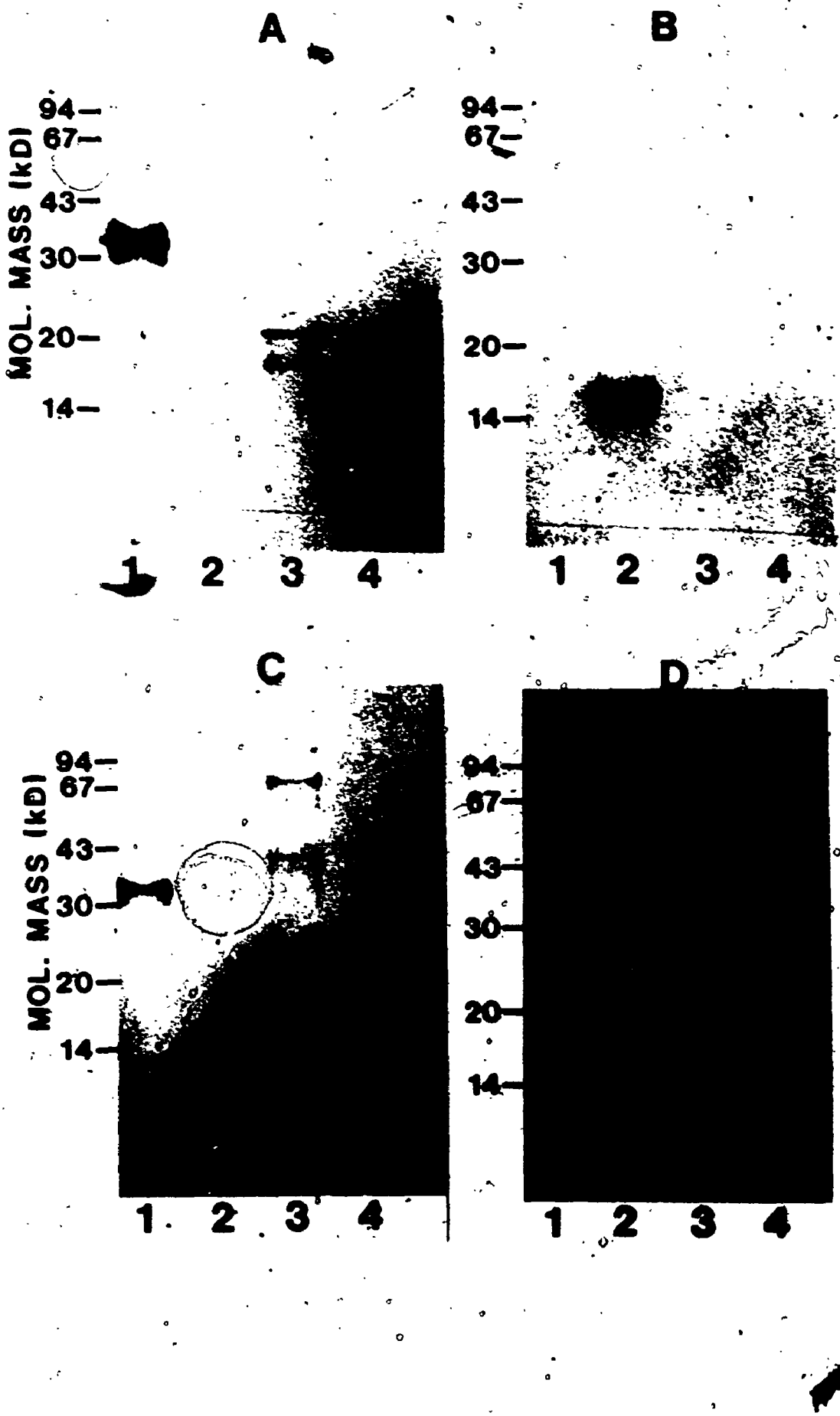


Figure 20. Collage of Western blots showing reactivities and cross-reactivities of antisera with various sclerotial polypeptides. Crude sclerotial polypeptides of M. borealis (W51), lane 1, C. psychromorbidus (LRS131), lane 2, T. idahoensis (W21), lane 3, and T. incarnata (W29), lane 4 respectively were blotted onto membranes A, B, C and D. Blots A, B, C and D were probed with antisera raised against the major sclerotial polypeptides of M. borealis (W51), C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) respectively. Numbers in the left margins indicate molecular standards.


A
B
C
D
MOL. MASS (kD)
94
67
43
30
20
14
1 2 3 4

polypeptides of C. psychromorbidus (LRS131) reacted positively with only the major (12.9-14.5 kD) sclerotial polypeptides of C. psychromorbidus (LRS131) (Fig. 20B, lane 2). No cross-reaction was observed against any sclerotial polypeptides in M. borealis (W51), T. idahoensis (W21) and T. incarnata (W29) (Fig. 20B, lanes 1, 3 and 4 respectively). This was also totally consistent with the Ouchterlony results.

Antiserum raised against the major sclerotial polypeptides of T. idahoensis (W21) reacted positively with the major low (16.2-21.9 kD) molecular mass sclerotial polypeptides of T. idahoensis (W21) (Fig. 20C, lane 3). The antiserum also cross-reacted with the major high (72.4 kD) molecular mass sclerotial polypeptides of T. idahoensis (W21). This was consistent with the Ouchterlony data previously described. In addition, cross-reactions were observed with the major (33.9 kD) sclerotial polypeptide of M. borealis (W51) (Fig. 20C, lane 1) and with the major (12.9-14.5 kD) sclerotial polypeptides of C. psychromorbidus (LRS131) (Fig. 20C, lane 2). No cross-reaction was observed with any of the sclerotial polypeptides of T. incarnata (W29) (Fig. 20C, lane 4). This was consistent with the Ouchterlony results presented earlier. The strong cross-reaction of W21 antiserum with the major sclerotial polypeptide of M. borealis (W51) (Fig. 20C, lane 1) was the only positive cross-reaction not observed using Ouchterlony double-diffusion.

Antiserum raised against the major (17.0 kD) and minor (18.6 kD) sclerotial polypeptides of T. incarnata (W29) reacted positively against the major and minor sclerotial polypeptides of T. incarnata (W29) (Fig. 20D, lane 4). Moreover, weak cross-reactions were observed with the major (12.9-14.5 kD) sclerotial polypeptides of C. psychromorbidus (LRS131) (Fig. 20D, lane 2) and with one of the major low molecular mass sclerotial polypeptides of T. idahoensis (W21) in the 16.2-21.9 kD range (Fig. 20D, lane 3). No cross-reaction was observed with any sclerotial polypeptides of M. borealis (W51) (Fig. 20D, lane 1). These results were also consistent with the Ouchterlony data.

In summary, all antisera reacted intensely with the major sclerotial polypeptide(s) against which they were raised. All observed cross-reactivities were consistent with Ouchterlony results except that W21 antiserum cross-reacted with the major (33.9 kD) sclerotial polypeptide of M. borealis (W51). This cross-reaction was the only one not observed using Ouchterlony double-diffusion. Furthermore, a total of 15 out of 16 Western blot treatments were consistent with their respective Ouchterlony data.

3.4 DISCUSSION

The results clearly indicate that the major sclerotial

polypeptides can be purified successfully by SDS-PAGE followed by electroelution. Similar procedures have been employed in the study of subunits of propionyl-CoA carboxylase (Kalousek et al., 1980) and heat shock proteins (Kelley and Schlesinger, 1982; Baszczynski, 1986). The presence of polypeptide bands in the 36-40 kD range, in the lane containing purified low molecular mass sclerotial polypeptides (16.2-21.9 kD) of W21 (Fig. 11), probably resulted from intermolecular disulfide bond formation during electroelution. This is supported by a quantitative decrease in the amount of these aggregates in response to increasing concentrations of β-mercaptoethanol, a strong reducing agent. In addition, a corresponding increase in the quantity of the purified low molecular weight sclerotial polypeptides of T. idahoensis was observed in response to aggregate reduction. Furthermore, these aggregates had a molecular mass (36-40 kD) approximately twice that of the purified lower molecular mass sclerotial polypeptides.

The summary of the Ouchterlony immunodiffusion reactions presented in Table V indicated that all antisera reacted positively with their respective antigens and also with other purified sclerotial polypeptides. Western blot data verified these results. Thus, a certain degree of structural similarity exists among the purified sclerotial polypeptides of varying molecular mass.

In a similar manner, Petersen et al. (1982) have shown

antigenic relatedness among certain major sclerotial proteins of mesophilic Sclerotinia species. Presently, it has been demonstrated that LRS131 antiserum raised against the major low molecular mass sclerotial polypeptides of Coprinus psychromorbidus (LRS131), a psychrophile, cross-reacted with a crude sclerotial protein extract of Sclerotium rolfsii, a mesophilic species. It was demonstrated previously that sclerotia of S. rolfsii contained a major polypeptide in the same molecular weight range as those found in the sclerotia of C. psychromorbidus (Insell et al., 1985). Thus, it has been shown that sclerotial protein(s) of S. rolfsii not only exhibit a similar molecular mass but are also antigenically similar to the sclerotial proteins of C. psychromorbidus (LRS131). Therefore, sclerotial proteins from psychrophilic and mesophilic fungi may exhibit a high degree of homology. This may be indicative of a common function for these polypeptides.

These studies have also shown that Ouchterlony double-diffusion and Western blot data were highly consistent. This observation corroborates the initial Ouchterlony work. However, it should be noted that both procedures have their respective advantages. Ouchterlony double-diffusion enables one to determine the degree of antigenic relatedness of proteins, whereas Western blotting enables one to determine how a particular antiserum is reacting with a complex mixture of proteins. For example, it was

clearly demonstrated in the Western blots that all antisera reacted strongly with their respective antigens, the major sclerotial polypeptides. In addition, it was shown that the antisera cross-reacted with some, but not all of the major sclerotial polypeptides from other species. None of the antisera cross-reacted with unidentified sclerotial polypeptides from the same species, thus confirming the specificity of the antisera. Furthermore, cross-reactions were observed only against other major sclerotial polypeptides and not against unidentified sclerotial polypeptides from other species. This provided conclusive evidence for the antigenic relatedness of the major sclerotial polypeptides of these psychrophilic fungi.

In this study, Ouchterlony double-diffusion data presented evidence that a major sclerotial polypeptide of cultured sclerotia of M. borealis (W51) was antigenically identical to a sclerotial polypeptide of field sclerotia of M. borealis. This provided further support for the reality of the major sclerotial polypeptide of cultured sclerotia of M. borealis (W51) and represented the second piece of corroborative evidence that the major sclerotial polypeptide of M. borealis (W51) was not an artifact of culturing conditions. More evidence in this regard will be discussed in Chapters 4 and 5.

However, the immunodiffusion data did indicate some unexpected relationships. First, anti LRS131 reacted only against its respective antigen, the purified sclerotial

polypeptide of LRS131. Yet, all other antisera cross-reacted with the purified sclerotial polypeptide of LRS131. Second, W51 antiserum cross-reacted with the purified sclerotial polypeptides of W21. However, W21 antiserum did not cross-react with the purified sclerotial polypeptide of W51. Third, W29 antiserum cross-reacted with the purified sclerotial polypeptides of W21, but the W21 antiserum did not cross-react with the purified sclerotial polypeptide of W29.

During the course of the immunodiffusion studies, it was observed that the antisera produced a positive reaction against their respective antigens much more rapidly (24 hours) when the antigen was denatured than against non-denatured antigen (more than 48 hours). From these data, it was concluded that the antigenic sites were either more accessible when the sclerotial proteins were in the denatured state or that the sclerotial proteins were structurally modified when in the non-denatured state. The former explanation was favoured since denatured proteins were used to produce the respective antibodies. Hence, the observed antigenic relationships may be due partially to the steric hindrance imposed by the conformational state of a given polypeptide on the accessibility of certain polyclonal antibodies to a particular antigenic site. Clearly, the antigenic domain associated with LRS131 sclerotial polypeptides is present in the major sclerotial polypeptides of all the psychrophilic species examined.

Furthermore, the LRS131 sclerotial polypeptides exhibited the smallest molecular mass (12.9-14.5 kD), and thus, should exhibit the least steric hindrance. This hypothesis is consistent with the immunological data regarding the cross-reactivity of anti LRS131 with sclerotial polypeptides of S. rolfsii and the lack of cross-reactivity with sclerotial polypeptides of S. sclerotiorum. Insell et al. (1985) have shown that sclerotia of S. rolfsii contain a major polypeptide of 16 kD, whereas sclerotia of S. sclerotiorum contained a major polypeptide of 36 kD. Clearly, further work is required to substantiate this hypothesis.

The cross-reactivity of the W21 antiserum against the purified high molecular mass (72.4 kD) sclerotial polypeptide of W21 indicated that one or more of the major low molecular mass (16.2-21.9 kD) sclerotial polypeptides of W21 possessed an antigenic determinant identical to the high molecular mass sclerotial polypeptide of W21. Possibly, one or more of the major low molecular mass (16.2-21.9 kD) sclerotial polypeptides resulted from post-translational cleavages of the high molecular mass (72.4 kD) sclerotial polypeptides. Evidence for this phenomenon was also alluded to previously. In Chapter 2, it was reported that the high molecular mass sclerotial polypeptide of W21 accumulated in the sclerotial initials of W21. Subsequently, the content of this polypeptide decreased in the mature sclerotia of W21. This was

accompanied by a corresponding increase in the quantity of the major low molecular mass sclerotial polypeptides in the mature sclerotia of W21 which possessed an antigenic determinant identical with the high molecular mass sclerotial polypeptide of W21.

The experiments and analyses presented in this chapter establish that immunological relatedness exists among the major sclerotial polypeptides of psychrophilic and mesophilic fungi. The variability observed in the amount of cross-reactivity may indicate that the sclerotial polypeptides have similar, but not identical structural homologies. Alternatively, it may suggest the existence of overlapping antigenic sites (Getzoff et al., 1987). Lastly, the structural relatedness of these polypeptides indicates that they may share a similar function. One possibility is that they are storage proteins. The next chapter furnishes evidence to support this hypothesis.

CHAPTER 4

IMMUNOCYTOCHEMICAL LOCALIZATION OF THE MAJOR SCLEROTIAL POLYPEPTIDES AND HISTOCHEMICAL STAINING

4.1 INTRODUCTION

In Chapter 2, it was observed that sclerotial development in M. borealis (W51), C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) was characterized by the accumulation of major polypeptides which can constitute up to 60% of the total polypeptide complement. Subsequently, polyclonal antibodies were produced against these major sclerotial polypeptides. In this chapter, the antibodies raised against the major sclerotial polypeptides are exploited for the purpose of localizing the major sclerotial polypeptides in the mature sclerotia of the four snow molds being examined. This is important since the localization of a particular protein may shed some light on the function of that protein. Furthermore, the following studies present the first unequivocal evidence for the localization of the major sclerotial polypeptides within protein bodies of mature sclerotia of psychrophilic fungi using the technique of immunofluorescence. This observation is supported by corroborative histochemical data on protein staining. Polysaccharide and lipid staining are also described.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of tissue.

Snow mold cultures were grown as previously described (Chapter 2) to produce sclerotial initials and mature sclerotia of each species. In preparation for light microscopy, mature sclerotia and sclerotial initials were infiltrated with 2.6% (w/v) glutaraldehyde in 0.02 M Na-phosphate buffer (pH 7.0) containing 0.3 M sucrose as osmoticum, and fixed for 3 hours at 25°C. After rinsing three times in cold 0.02 M Na-phosphate buffer (pH 7.0) containing 0.3M sucrose, the tissues were post-fixed at room temperature with 1% (w/v) osmium tetroxide in 0.02 M Na-phosphate buffer (pH 7.0) containing 0.3 M sucrose. They were then rinsed with 0.02 M Na-phosphate buffer containing 0.3 M sucrose, followed by several rinses with distilled water. The tissues were then dehydrated in a graded series of acetone. Tissues were infiltrated and embedded in Spurr's epoxy resin (J.B. EM Services Inc., Pointe Claire - Dorval, Quebec, Canada). Spurr's was chosen because of its low viscosity and good infiltration characteristics. Spurr's embedding medium was prepared by gravimetrically adding the following components into a plastic cup: vinylcyclohexene dioxide, 10 g; diglycidyl ether of polypropylene glycol, 6 g; nonenyl succinic anhydride, 26 g; dimethylaminoethanol, 0.4 g. The catalyst, dimethylaminoethanol, was added last after gently

mixing the other components. Samples were allowed to polymerize for 8 hours in an oven set at 70°C. Once polymerized, the blocks were trimmed and sections (0.5 µm thick) were cut with glass knives on a Reichert Ultracut E ultramicrotome, mounted onto glass slides and heat-affixed for 5 minutes at 80°C.

4.2.2 Histochemical staining procedures.

Proteins were stained with 1% (w/v) Aniline Blue Black (C.I. 20470) in 7% (v/v) acetic acid or with 0.25% Coomassie Brilliant Blue (C.I. 42660) in 7% (v/v) acetic acid for 10 minutes at 50-60°C according to Fisher (1968). Sections were rinsed, dried and mounted in DPX mountant.

Polysaccharides were oxidized for 10 minutes in 0.04 M periodic acid, rinsed with distilled H_2O and then stained for 10 minutes with 1% (w/v) Schiff reagent according to Lillie (1956). Sections were rinsed thoroughly with 0.5% (w/v) sodium metabisulfite, followed by rinsing with distilled H_2O. Sections were then dried and mounted in DPX mountant.

Lipids were stained for 20-30 minutes with 0.05% (w/v) Nile blue in 1% (v/v) concentrated H_2SO_4 according to Lillie (1956). Sections were rinsed, dried and mounted in DPX mountant.

4.2.3 Immunocytochemical labelling.

For immunofluorescent localization of sclerotial polypeptides, an indirect method similar to the one of Bendayan (1984) was used. First, thick tissue sections were immersed in a saturated solution of sodium metaperiodate for 2 hours, thoroughly but gently washed with distilled H_2O and air dried. Next, 10-15 µl of diluted primary antiserum (1:20 with 0.01 M phosphate-buffered saline [PBS] pH 7.0) was pipetted over the section on the glass slide. The slides were placed under petri dish lids to prevent desiccation and incubated for 60-90 minutes. The sections were lightly rinsed with PBS to remove excess unbound antibodies and while the sections were still damp, 20 µl of a 0.3 mg/ml solution of fluoroscein isothiocyanate (FITC) conjugated to protein A (Boehringer Mannheim, Dorval, Quebec, Can.) was applied to the sections which were then incubated under petri dish lids for 60-90 minutes. Following incubation, the sections were gently rinsed with PBS and distilled water and dried. They were then mounted in PBS-glycerol (1:4, v/v) containing 0.01% (w/v) p-phenylenediamine (Aldrich Chemical Company, Milwaukee, Wis., USA), which retards the fading of fluorescence during microscopy (Johnson and de C. Nogueira-Araujo, 1981; Reed and Chollet, 1985). The sections were observed and photographed with a Leitz Orthoplan photomicroscope equipped with epifluorescence. The excitation filter used was a 12/3 filter for FITC.

4.3 RESULTS

4.3.1 Protein staining.

Protein bodies were revealed in the sclerotia of C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) after staining with Aniline Blue or Coomassie Blue (Figs. 21, 24, 27, 33, 39, 41 and 45). Protein bodies were not observed in either sclerotial initials or hyphae of these species, rather they exhibited the presence of amorphous proteinaceous material (Figs. 34, 43 and 47).

In C. psychromorbidus (LRS131), copious protein bodies were evident and varied from 0.2 µm to about 3.0 µm in size (Figs. 21 and 33). They appeared to be uniformly distributed throughout the cortical and medullary cells but were not evident in the rind cells of the sclerotia (Fig. 22).

Protein bodies of T. idahoensis (W21) showed less variation in size, ranging from about 0.2 µm to 1.2 µm (Figs. 24, 39 and 41). Generally, they were densely clustered throughout all the sclerotial cells but appeared to be more numerous in the rind cells (Figs. 24 and 41). In contrast, protein bodies of T. incarnata (W29) were more numerous in the cortical and medullary cells (Fig. 27). Generally, protein bodies in the sclerotial cells of T. incarnata (W29) were less numerous than those of T.

Figure 21. Cross-section of a mature sclerotium of C. psychromorbidus (LRS131) stained with Coomassie Blue R. Arrows indicate protein bodies. Bar equals 5 µm.

Figure 22. Cross-section of a mature sclerotium of C. psychromorbidus (LRS131) treated with LRS131 antiserum and then labelled with protein A-FITC. R, rind. Arrows indicate protein bodies. Bar equals 5 µm.

Figure 23. Cross-section of a mature sclerotium of C. psychromorbidus (LRS131) treated with pre-immune serum and then labelled with protein A-FITC. Arrow indicates cell wall. M, extracellular matrix; R, rind. Bar equals 5 µm.

Figure 24. Cross-section of a mature sclerotium of T. idahoensis (W21) stained with PAS and then counterstained with Coomassie Blue R. Arrows indicate protein bodies. R, rind. Bar equals 5 µm.

Figure 25. Cross-section of a mature sclerotium of T. idahoensis (W21) treated with W21 antiserum and then labelled with protein A-FITC. Arrows indicate protein bodies. R, rind. Bar equals 5 µm.

Figure 26. Cross-section of a mature sclerotium of T. idahoensis (W21) treated with pre-immune serum and then labelled with protein A-FITC. R, rind. Arrow indicates cell wall. Bar equals 5 µm.

Figure 27. Cross-section of a mature sclerotium of T. incarnata (W29) stained with PAS and then counterstained with Coomassie Blue R. Arrows indicate protein bodies. R, rind; CW, cell wall; asterisk, extracellular matrix. Bar equals 5 µm.

Figure 28. Cross-section of a mature sclerotium of T. incarnata (W29) treated with W29 antiserum and then labelled with protein A-FITC. Arrows indicate protein bodies. Bar equals 5 µm.

Figure 29. Cross-section of mature sclerotium of T. incarnata (W29) treated with pre-immune serum and then labelled with protein A-FITC. Arrow indicates cell wall. Bar equals 5 µm.

Figure 30. Cross-section of a mature sclerotium of M. borealis (W51) stained with PAS and then counterstained with Coomassie Blue R. Arrows indicate amorphous proteinaceous material in the cytoplasmic peripheries. R, rind; M, extracellular matrix. Bar equals 5 µm.

Figure 31. Cross-section of a mature sclerotium of M. borealis (W51) treated with W51 antiserum and then labelled with protein A-FITC. Arrows indicate amorphous proteinaceous material localized in the cytoplasmic peripheries. R, rind; M, extracellular matrix. Bar equals 5 µm.

Figure 32. Cross-section of a mature sclerotium of M. borealis (W51) treated with pre-immune serum and then labelled with protein A-FITC. Arrow indicates cell wall. Bar equals 5 µm.

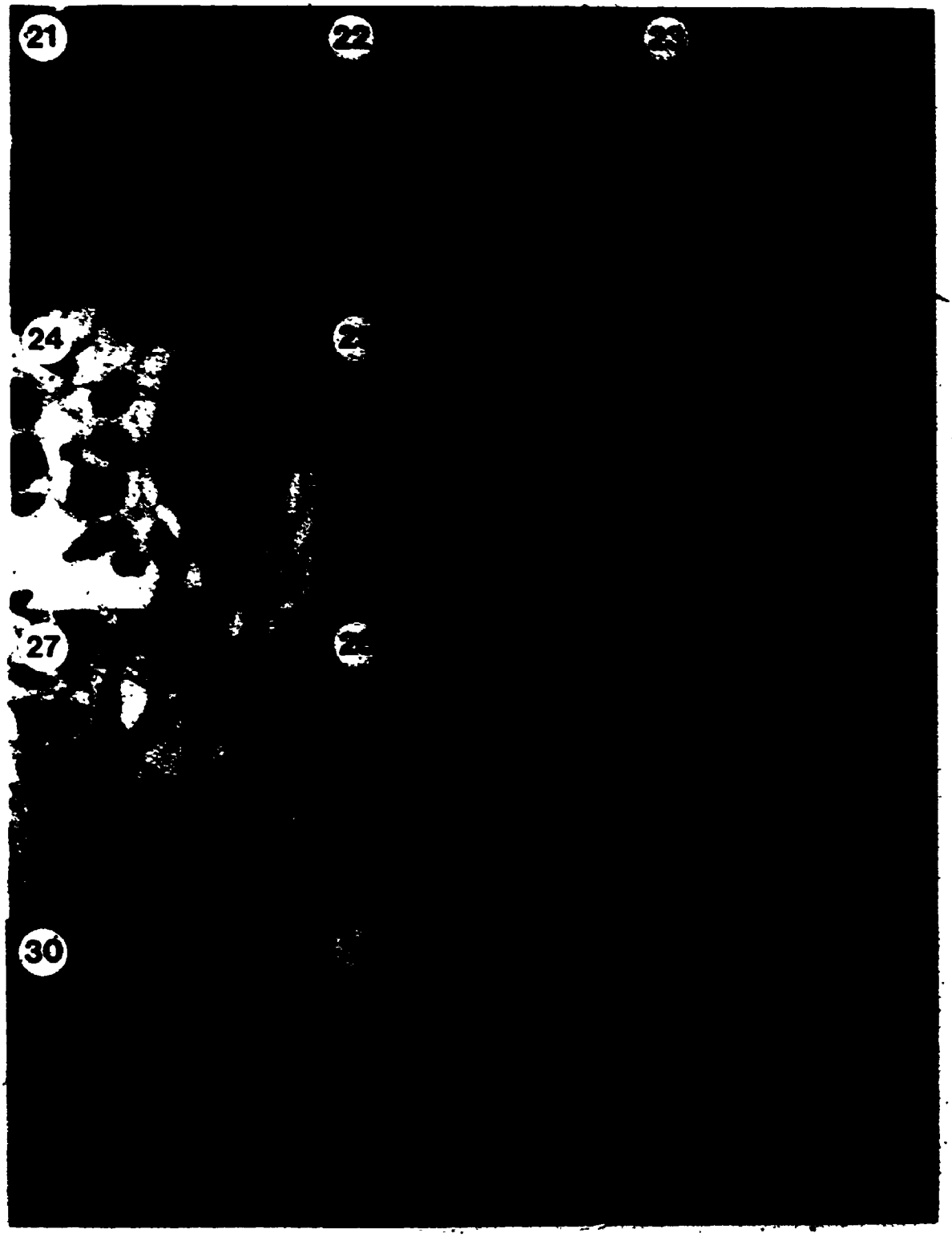

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Figure 33. Cross-section of a mature sclerotium of C. psychromorbidus (LRS131) stained with Aniline Blue Black. PB, protein bodies. Bar equals 5 µm.

Figure 34. Cross-section of a sclerotial initial of C. psychromorbidus (LRS131) stained with Aniline Blue Black. AP, amorphous proteinaceous material; LD, refractile lipid droplets. Bar equals 5 µm.

Figure 35. Cross-section of a mature sclerotium of C. psychromorbidus (LRS131) stained with PAS. CP, cytoplasmic polysaccharide material; CW, cell wall; asterisk, extracellular matrix. Bar equals 5 µm.

Figure 36. Cross-section of a sclerotial initial of C. psychromorbidus (LRS131) stained with PAS. CP, cytoplasmic polysaccharide material; CW, cell wall; LD, refractile lipid droplets; asterisk, extracellular matrix. Bar equals 5 µm.

Figure 37. Cross-section of a mature sclerotium of C. psychromorbidus (LRS131) stained with Sulfuric Nile Blue. LD, lipid droplets; PB, nonstaining protein bodies; asterisk, extracellular matrix. Bar equals 5 µm.

Figure 38. Cross-section of a sclerotial initial of C. psychromorbidus (LRS131) stained with Sulfuric Nile Blue. LD, lipid droplet. Bar equals 5 µm.

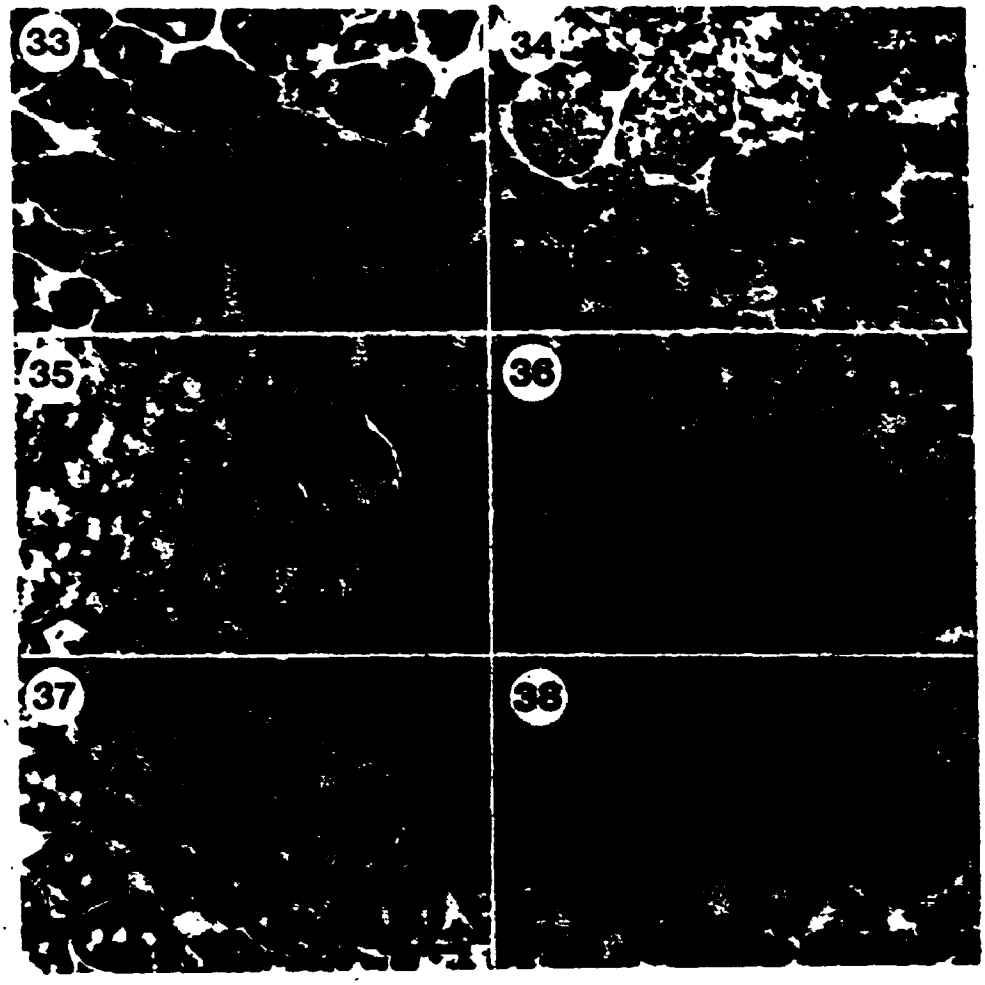
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Figure 39. Cross-section of a mature sclerotium of T. idahoensis (W21) stained with PAS and then counterstained with Aniline Blue Black. PB, protein bodies. Bar equals 5 µm.

Figure 40. Serial-section of a mature sclerotium (Fig. 39) of T. idahoensis (W21) stained only with PAS. CP, cytoplasmic polysaccharide material; PB, nonstaining protein bodies; asterisk, extracelluiar matrix. Bar equals 5 µm.

Figure 41. Cross-section of a mature sclerotium of T. idahoensis (W21) stained with PAS and then counterstained with Coomassie Blue R. PB, protein bodies; R, rind. Bar equals 5 µm.

Figure 42. Cross-section of a mature sclerotium of T. idahoensis (W21) stained with Sulfuric Nile Blue. R, rind. Arrows indicate thickness (approximately 3.0 µm) of the outer surface layer of rind. CW, cell walls of extrasclerotial hyphae. Bar equals 5 µm.

Figure 43. Cross-section of a sclerotial initial of T. idahoensis (W21) stained with Coomassie Blue R. AP, amorphous proteinaceous material. Arrows indicate the outer surface of the developing rind. Bar equals 5 µm.

Figure 44. Cross-section of a sclerotial initial of T. idahoensis (W21) stained with PAS. CW, cell wall. Arrows indicate a thickened layer of polysaccharide material on the outer surface of the developing rind cells. Bar equals 5 µm.

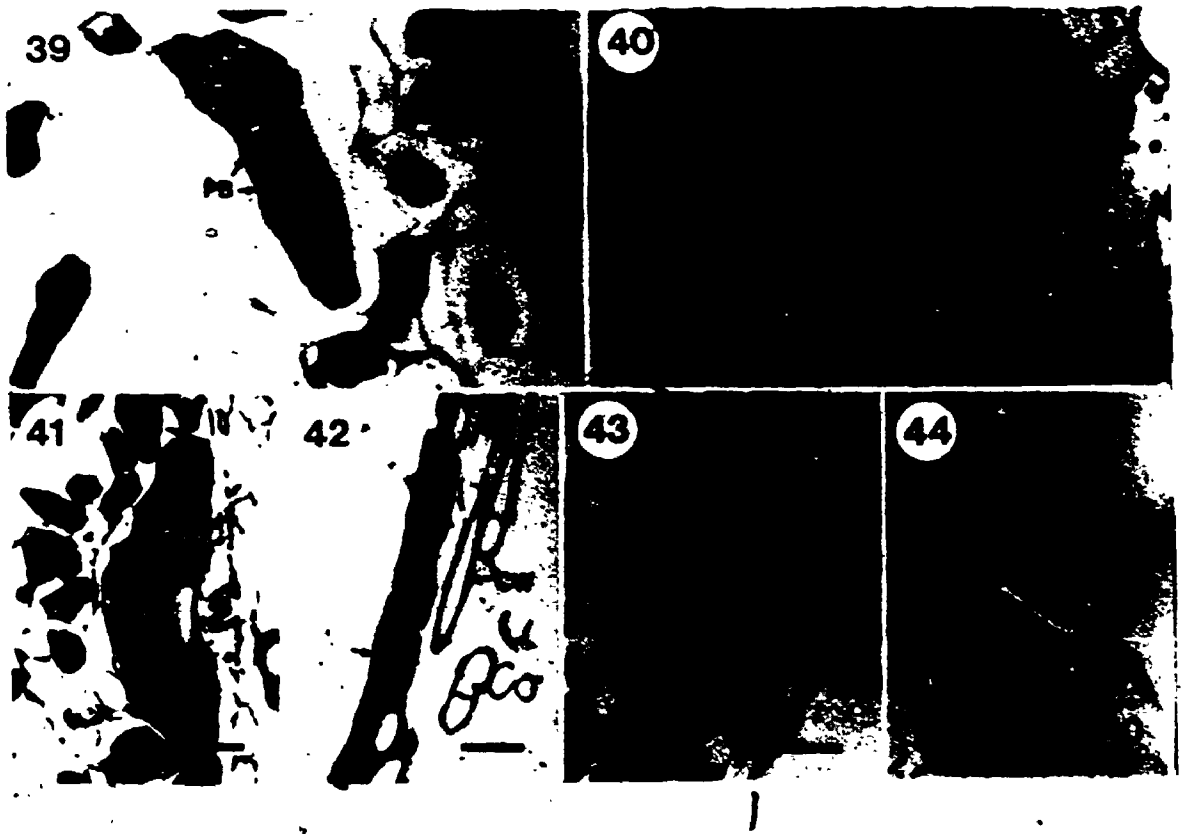
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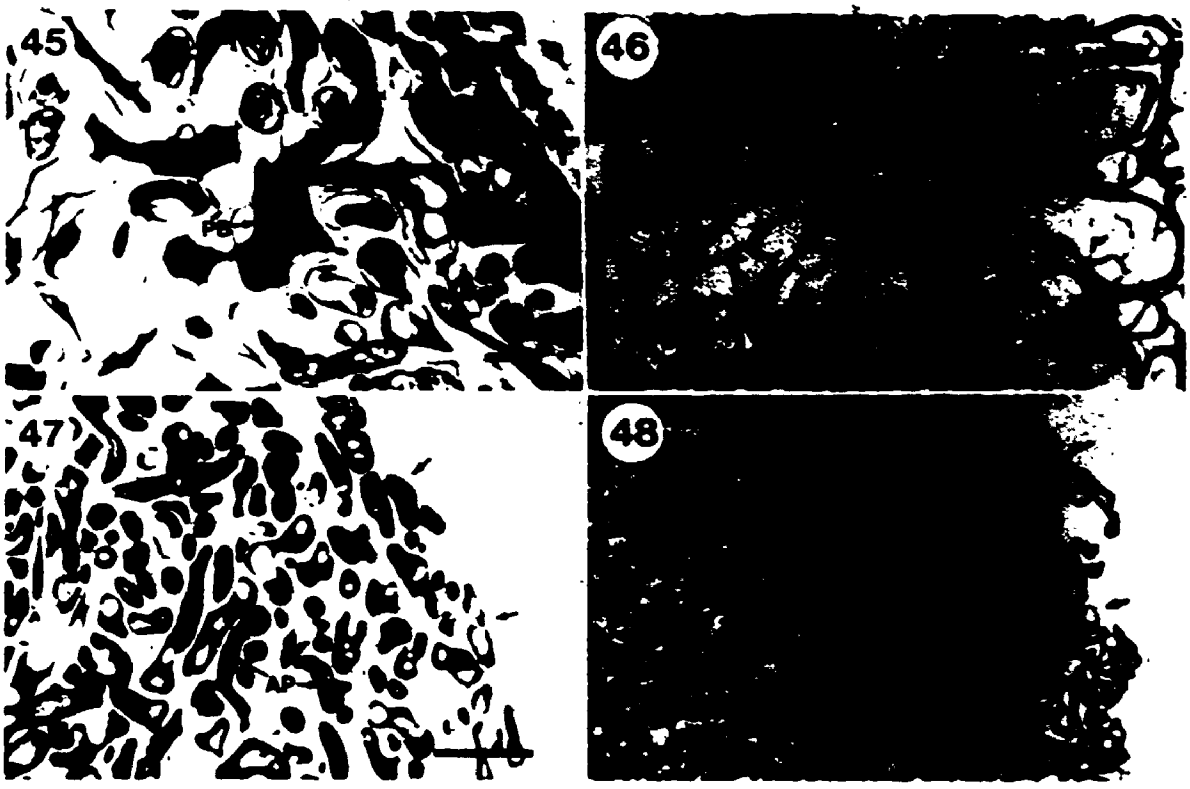
Figure 45. Cross-section of a sclerotium of T. incarnata (W29) stained with PAS and then counter-stained with Coomassie Blue R. PB, protein body; CP, cytoplasmic polysaccharide material; R, rind. Bar equals 5 µm.

Figure 46. Cross-section of a sclerotium of T. incarnata (W29) stained with PAS. CP, cytoplasmic polysaccharide material; CW, cell wall; asterisk, extracellular matrix. Bar equals 5 µm.

Figure 47. Cross-section of a sclerotial initial of T. incarnata (W29) stained with Coomassie Blue R. AP, amorphous proteinaceous material. Arrows indicate the outer surface of the developing rind. Bar equals 10 µm.

Figure 48. Cross-section of a sclerotial initial of T. incarnata (W29) stained with PAS. CP, cytoplasmic polysaccharide material; CW, cell wall. Arrows indicate the outer surface of the developing rind. Bar equals 20 µm.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idahoensis (W21), but some appeared larger, ranging from 1.0 µm to 1.5 µm in diameter (compare Figs. 27 and 24).

In contrast to the other three species examined, cultured sclerotia of M. borealis (W51) showed no evidence of distinct protein bodies. Rather, amorphous proteinaceous accumulations were observed predominantly in the cytoplasmic peripheries of the rind and cortical cells of cultured sclerotia (Figs. 30 and 49). Amorphous protein was also observed in the sclerotial initials of W51 (Fig. 51).

4.3.2 Immunofluorescence.

The results in Figs. 23, 25 and 28 indicate that the immunofluorescence is specifically associated with distinct cytoplasmic inclusions which are similar in size, number and cellular distribution to the protein bodies visualized histochemically in the mature sclerotia of C. psychromorbidus (LRS131), T. idahoensis (W21), and T. incarnata (W29). Neither protein A-FITC conjugated to pre-immune serum (Figs. 23, 26, 29 and 32) nor protein A-FITC treatment alone caused these protein bodies to fluoresce. Yellow autofluorescence was generally associated with cell walls and extracellular matrices.

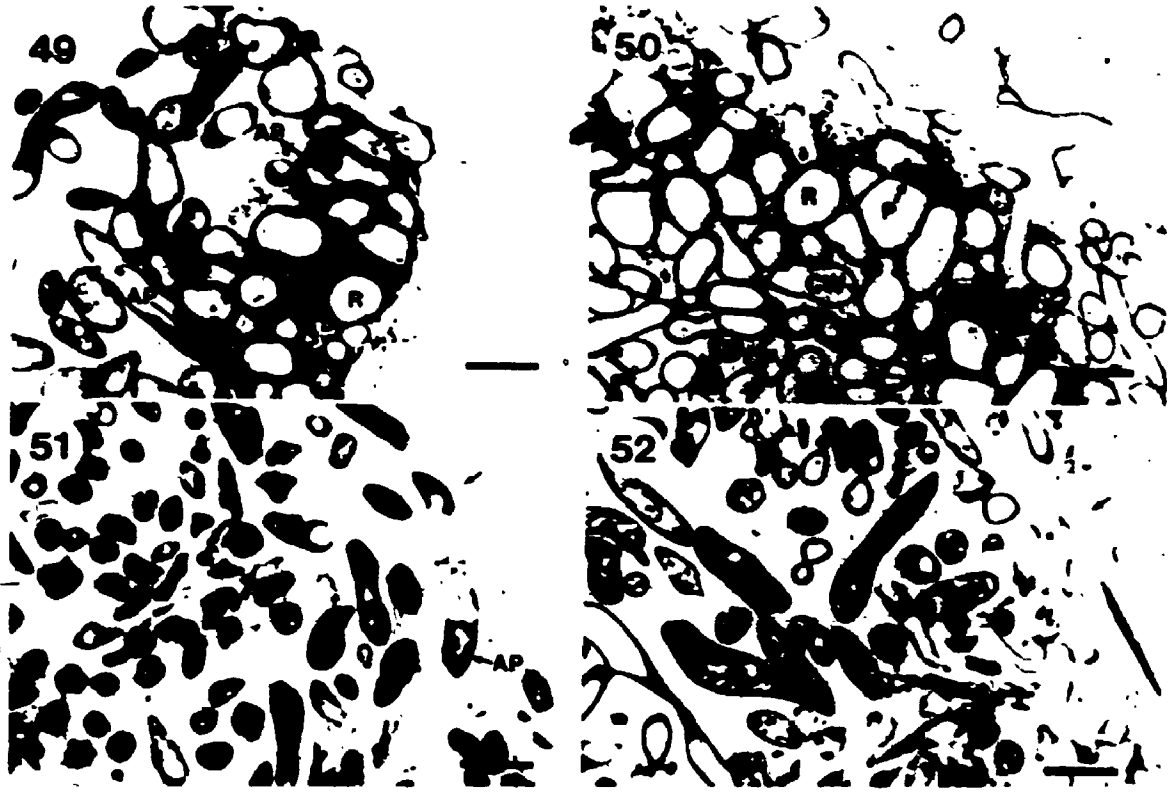
Sclerotia of M. borealis (W51) exhibited several unique labelling characteristics. In contrast to the other species, most of the immunofluorescence present in cultured sclerotia of W51 appeared to be associated with the

Figure 49. Cross-section of a mature sclerotium of M. borealis (W51) stained with PAS and then counterstained with Coomassie Blue R. AP, amorphous proteinaceous material; R, rind. Bar equals 5 µm.

Figure 50. Cross-section of a mature sclerotium of M. borealis (W51) stained with PAS. CP, cytoplasmic polysaccharide material; CW, cell wall; R, rind; P, dark, nonstaining material. Bar equals 5 µ.

Figure 51. Cross-section of a sclerotial initial of M. borealis (W51) stained with Coomassie Blue R. AP, amorphous proteinaceous material. Arrow indicates the outer surface of the developing rind. Bar equals 5 µm.

Figure 52. Cross-section of a sclerotial initial of M. borealis (W51) stained with PAS. CP, cytoplasmic polysaccharide material. Arrow indicates the outer surface of the developing rind. Bar equals 5 µm.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51
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AP

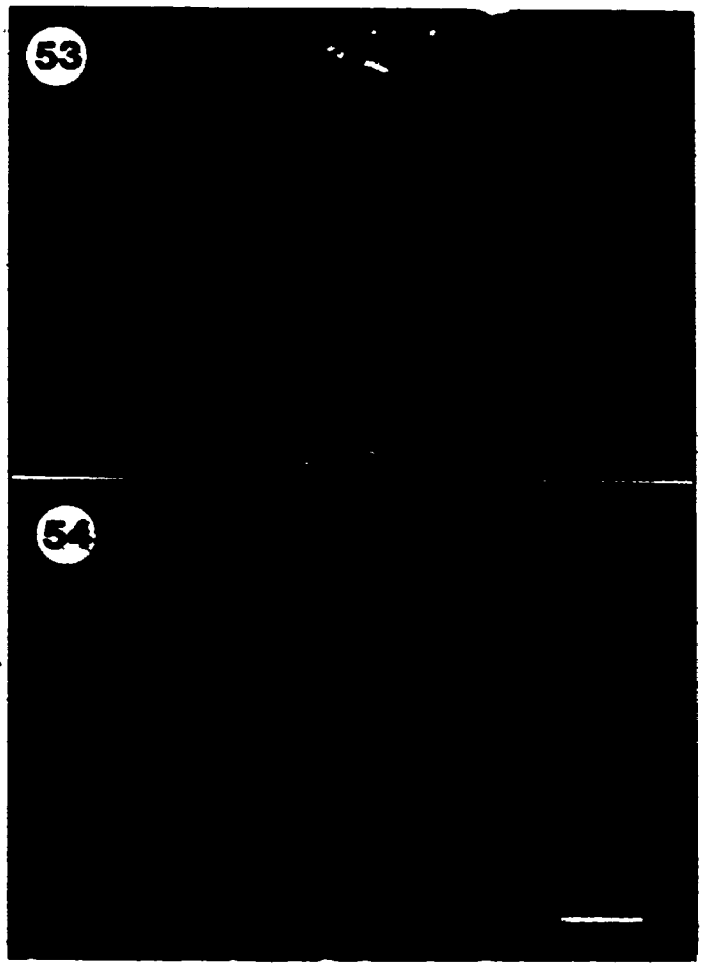
cytoplasmic peripheries of rind and cortical cells rather than with distinct protein bodies (Fig. 31). This is consistent with the amorphous proteinaceous accumulations observed histochemically (Fig. 30). However, field grown sclerotia of M. borealis did exhibit immunofluorescence specifically associated with distinct intracellular inclusions which ranged in size from 0.5 µm to 1.0 µm in diameter (Fig. 53). In contrast, no fluorescing bodies were observed in sections of field sclerotia treated with protein A-FITC conjugated to pre-immune serum (Fig. 54). Thus, the immunofluorescence data not only complement the histochemical data but, more importantly, indicate that the major sclerotial polypeptides of C. psychromorbidus (LRS131), T. idahoensis (W21), T. incarnata (W29) and field grown sclerotia of M. borealis are specifically localized within intracellular protein bodies.

4.3.3 Polysaccharide staining.

Mature sclerotia of all species examined stained positively for the presence of polysaccharides (Figs. 35, 40, 46 and 50). Staining was generally associated with the cell walls and extracellular matrices of mature sclerotia of all four species. Cytoplasmic polysaccharide material was also present in the cortical and medullary cells of C. psychromorbidus (LRS131), T. idahoensis (W21), T. incarnata (W29) and M. borealis (W51) (Figs. 35, 40, 46 and 50 respectively).

Figure 53. Cross-section of a mature field sclerotium of M. borealis treated with W51 antiserum and then labelled with protein A-FITC. PB, fluorescing protein bodies. Bar equals 5 µm.

Figure 54. Cross-section of a mature field sclerotium of M. borealis treated with pre-immune serum (control) and then labelled with protein A-FITC. Arrows indicate autofluorescence in cell walls. Bar equals 5 µm.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The cell walls and extracellular matrices of sclerotial initials stained positively with the Schiff reagent (Figs. 34, 44, 48 and 52). In addition, sclerotial initials of T. incarnata (W29) and M. borealis (W51) exhibited the presence of amorphous, cytoplasmic material which stained positively for polysaccharides (Figs. 48 and 52 respectively).

4.3.4 Lipid staining.

Cortical and medullary cells of the sclerotia of C. psychromorbidus (LRS131) contained copious droplets which were stained blue with Sulfuric Nile Blue (Fig. 37). These structures were generally 1 µm to 1.4 µm in diameter. Extracellular matrices were also stained blue (Fig. 37). Protein bodies appeared as unstained structures while extracellular matrices stained intensely (Fig. 37). Lipid droplets were also observed in the cortical and medullary cells of the sclerotial initials of C. psychromorbidus (LRS131) (Fig. 38).

A layer of intensely stained material (3.0 µm thick) was observed on the outer surface of the sclerotial rind of T. idahoensis (W21) (Fig. 42) and T. incarnata (W29) (data not shown). Cell walls of some extra-sclerotial hyphae of W21 also stained intensely (Fig. 42). Little evidence of staining with Sulfuric Nile Blue occurred in the sclerotial initials of T. idahoensis (W21) and T. incarnata (W29) where the rind was just beginning to develop (data not

shown). No lipid reserves were detected in the sclerotia or sclerotial initials of M. borealis (W51).

4.4 DISCUSSION

Protein bodies were observed in the mature sclerotia of C. psychromorbidus, T. idahoensis and T. incarnata using protein stains. Using immunofluorescence microscopy, it has been shown for the first time that the major sclerotial polypeptides of these species are sequestered in these protein bodies. In contrast to mature sclerotia, protein bodies were not detected in the sclerotial initials of these species, only amorphous proteinaceous material in the cytoplasm. In the sclerotial initials, polypeptides of molecular mass similar to those of the major sclerotial polypeptides were usually observed by electrophoresis (Chapter 2). These results may indicate that the major sclerotial polypeptides have been synthesized early in sclerotial differentiation and subsequently packaged into protein bodies upon sclerotial maturation. This is consistent with the recent work of Grenville et al. (1985a, 1985b) who noted the presence of distinct protein inclusions during sclerotial development in two ectomycorrhizal fungi, Paxillus involutus and Pisolithus tinctorius. In addition, the ultrastructure of Sclerotinia minor, a mesophile, has been examined by Bullock et al. (1980a, 1980b; 1983). In this study, they were also able

to show the presence of protein bodies in the sclerotia of this species.

Histochemical protein staining and immunofluorescence of M. borealis indicated a difference between cultured and field grown sclerotia. Although cultured and field grown sclerotia exhibited the presence of major polypeptides which were of the same molecular mass (Chapter 2) and were immunologically identical, these polypeptides were present as amorphous proteinaceous inclusions in cultured sclerotia but were localized in distinct protein bodies in field grown sclerotia. This discrepancy could be an artifact of culturing conditions and/or an artifact of differential stability of the protein bodies of cultured versus field grown sclerotia to the fixation procedure. Alternatively, the discrepancy observed could be a reflection of the developmental age of the cultured sclerotia. There are insufficient data to suggest which of these explanations is most appropriate. Recently, however, the presence of protein bodies has been shown in cultured sclerotia of M. borealis (W51) (L. Kohn, personal communication).

Protein storage bodies have also been well documented in seeds (Lott, 1980; Spitzer and Lott, 1982). Thus, it may be possible that the major proteins sequestered in the protein bodies of the sclerotia of psychrophilic fungi function in a similar manner to those found in seeds of plants. This seems plausible since sclerotia contain protein bodies and represent perennating structures

analogous to seeds in plants. Russo et al. (1982) suggested that the major sclerotial protein in Sclerotinia sclerotiorum may act as a storage protein, that is, as a nitrogen source or as a source of amino acids for the synthesis of new proteins during carpogenic germination. A decreased level of the major sclerotial polypeptide in sclerotia after carpogenic germination supported this hypothesis (Russo et al., 1982). These data are corroborated by the ultrastructural work of Bullock et al. (1983) who showed evidence for the translocation of hydrolysis products from protein bodies in the sclerotium to the apothecial stipe during carpogenic germination in Sclerotinia minor. In addition, the recent discovery that the major sclerotial polypeptide of S. sclerotiorum is sequestered in protein bodies further strengthened their hypothesis (Russo and Van Etten, 1985). Based on the observations presented in this chapter, it is tempting to suggest that the major sclerotial polypeptides of snow molds function in a similar way. However, it is also possible that the major sclerotial polypeptides play a role in maintaining dormancy (Russo et al., 1982) or may have some unknown catalytic function. At this point, the first explanation is favoured but the latter two possibilities cannot be ruled out. Clearly, further work needs to be done to determine the precise role of these proteins.

Polysaccharide deposits were found in all snow mold sclerotia, as revealed by areas of intense red staining

with PAS. It is probable that these deposits consist of glycogen since numerous authors have reported glycogen as a major reserve in sclerotia of other mesophilic fungi (Revel et al., 1960; Bracker, 1967; Saito, 1974; Waters et al., 1975a, 1975b; Bullock et al., 1980b).

Apart from proteins and polysaccharides, the other major storage reserve found in sclerotia of some snow mold species is lipid. Lipid has been reported as a major storage product in sclerotia of Sclerotium rolfsii (Chet et al., 1977) and Sclerotinia sclerotiorum (Calonge, 1970). Bullock et al. (1980b), however, were unable to detect lipid in the sclerotia of Sclerotinia minor. In this study, the presence of intracellular lipid bodies was specific for sclerotial initials and mature sclerotia of Coprinus psychromorbidus. However, lipid deposits were found on the outer surface of the rind cells of the two Typhula spp. In sclerotia, lipids can act as energy reserves which are subsequently respired during carpogenic germination (Coley-Smith and Cooke, 1971). This may be true of sclerotia of C. psychromorbidus.

CHAPTER 5

WESTERN BLOT ANALYSIS OF THE ACCUMULATION OF THE MAJOR SCLEROTIAL POLYPEPTIDES IN VEGETATIVE HYPHAE AT PERMISSIVE AND NONPERMISSIVE TEMPERATURES

5.1 INTRODUCTION

High growth temperature has been implicated as an environmental factor which induces the differentiation of vegetative hyphae to mature sclerotia in snow molds (Hemmi and Endo, 1931; Dejardin and Ward, 1971a; Chet and Henis, 1975; Traquair et al., 1987). Since the major sclerotial polypeptides of several snow mold species appear to be associated specifically with sclerotial morphogenesis (Chapter 2), these gene products represent potential biochemical markers to examine the regulation of this differentiation process. Earlier, Newsted et al. (1985) showed that exposure of several sclerotial-forming snow mold species to nonpermissive growth temperatures (25°C) for prolonged periods of time resulted in the disappearance of all polypeptides within vegetative hyphae except for polypeptide(s) of molecular weights similar to the major polypeptides observed in mature sclerotia. In this chapter, antibodies raised to the major sclerotial polypeptides of M. borealis (W51), C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) are

utilized to probe for the presence of sclerotial polypeptides in vegetative hyphae subjected to permissive (5°C) and nonpermissive (25°C) growth temperatures. The purpose of this study was to determine unequivocally whether sclerotial polypeptides accumulate in vegetative hyphae subjected to nonpermissive growth temperatures. The relevance of these findings to temperature-induced differentiation in these fungi is discussed.

5.2 MATERIALS AND METHODS

5.2.1 Culturing and collection of hyphae.

Cultures of M. borealis (W51), C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) were grown in darkness in petri dishes on a defined agar medium at permissive temperature (5°C) until copious vegetative hyphae were formed on the surface of the agar. Subsequently, one set of plates from each species was transferred to nonpermissive temperature (25°C) for 14 days while another set from each species was left at permissive temperature (5°C) as the control set. At the end of 14 days, aerial hyphae from both the controls and the thermally shifted hyphae were collected. The hyphae were then placed in the receptacle of a glass micro-homogenizer (Radnoti) and kept on ice in preparation for protein extraction.

5.2.2 Protein extraction.

Vegetative hyphae were pulverized with a glass micro-homogenizer in 100 µl of sample buffer containing SDS and proteolytic inhibitors previously described in Chapter 2. The homogenates were then transferred to 1.5 ml centrifuge tubes and centrifuged for 5 minutes at 14,000 xg. The supernatants were then transferred to 0.5 ml centrifuge tubes. Protein content was determined using the turbidimetric method of Comings and Tack (1972) using trichloracetic acid as the protein precipitant. Samples were stored at -70°C until required.

5.2.3 Electrophoresis.

Samples consisting of 1 µg of extracted hyphal protein were loaded into wells of a one-dimensional SDS polyacrylamide gradient gel (12-20%) overlayed with a 5% stacking gel. In addition, crude extracts of sclerotial protein and standard proteins were loaded into separate wells to act as markers. The running buffer was the same as described in Chapter 2. The polypeptides were electrophoresed for 3 hours at 10°C at a constant current of 15 mA per gel using a Mighty Small II Slab Gel Electrophoresis Unit SE250 (Hoefer Scientic Instruments) equipped with a water-jacketed chamber with running tap water as coolant. Electrophoresis was continued until the bromophenol blue dye ran off the bottom of the resolving gel.

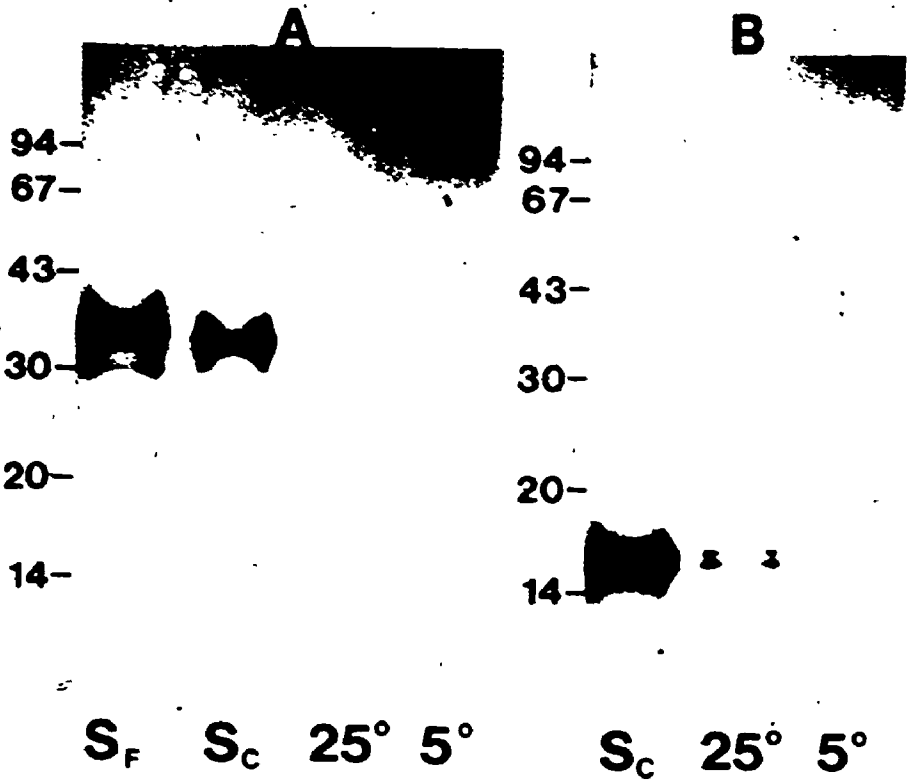
5.2.4 Electro-transfer and membrane processing.

At the end of the electrophoretic run, the slab gels were removed from the plates and prepared for electro-blotting. After electrophoretic transfer, the membranes were carefully removed from the gels. The sections onto which standard proteins had been transferred from the gels were stained while the rest of the nitrocellulose (or Immobilon) membranes, retaining the total polypeptide profiles of control and thermally-shifted vegetative hyphae, were washed four times in PBS-Tween. The membranes were then transferred to plastic petri dishes containing primary antibody solution. For each species, this consisted of a 1:2500 dilution of antiserum raised against the major sclerotial polypeptides in PBS-Tween. A detailed description of the electro-transfer and membrane processing protocol, as well as photography, is given in Chapter 3.

5.3 RESULTS

Antibodies raised against the major sclerotial polypeptide of M. borealis (W51) reacted positively with a single polypeptide band of the W51 vegetative hyphae which had been shifted to 25°C for 14 days (Fig. 55A). This band was in the 33 kD range which corresponded with major sclerotial polypeptide bands. In contrast, none of the antibodies raised against the major sclerotial polypeptide of M. borealis (W51) reacted positively with any of the

Figure 55. Collage of Western blots showing reactivities of antisera with polypeptides of vegetative hyphae subjected to different temperature treatments. A, B, C and D represent Western blots of polypeptides of M. borealis (W51), C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) respectively. Lane S_F, field sclerotia of M. borealis (W51); lanes S_C, cultured sclerotia; lanes 25°, vegetative hyphae shifted to 25 °C for 2 weeks; lanes 5°, vegetative hyphae incubated at 5°C. Western blots A, B, C and D were probed with antisera raised against the major sclerotial polypeptides of M. borealis (W51), C. psychromorbidus (LRS131), T. idahoensis (W21) and T. incarnata (W29) respectively. Numbers in the left margins indicate molecular weight standards.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B
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43
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20
14
94
67
43
30
20
14
S_F S_C 25° 5°
S_C 25° 5°
C
D
MOL. MASS (kD)
94
67
43
30
20
14
94
67
43
30
20
14
S_C 25° 5°
S_C 25° 5°

polypeptide bands of W51 control vegetative hyphae grown at 5°C. Antibodies also reacted positively with the major sclerotial polypeptide band of both the cultured and field sclerotia of M. borealis (Fig. 55A).

Antibodies raised against the major sclerotial polypeptides of C. psychromorbidus (LRS131) reacted positively with two polypeptide bands of LRS131 vegetative hyphae which had been shifted to 25°C for 14 days (Fig. 55B). These bands were in the 12.9-14.5 kD range and co-migrated with the major sclerotial polypeptide bands which also reacted positively with the antibodies (Fig. 55B). However, no reaction (Fig. 55B) was observed between the antibodies and any of the polypeptide bands of C. psychromorbidus (LRS131) control vegetative hyphae which were grown at 5°C.

Antibodies raised against the major sclerotial polypeptides of T. idahoensis (W21) reacted positively with three polypeptide bands (Fig. 55C) of W21 vegetative hyphae which had been shifted to 25°C for 14 days. The antibodies also reacted positively, but to a lesser degree, with three polypeptide bands of W21 control vegetative hyphae grown at 5°C (Fig. 55C). Furthermore, the three labelled bands of the control hyphae appeared to have the same molecular mass (16.2, 17.0 and 18.2 kD) as those observed in hyphae shifted to 25°C. Antibodies raised against the major sclerotial polypeptides of T. idahoensis (W21) reacted positively against 6 major low molecular weight sclerotial

polypeptide bands of cultured sclerotia. Three bands had the same molecular mass (16.2, 17.0 and 18.2 kD) as the bands observed in the vegetative hyphae. The other three sclerotial polypeptide bands had molecular masses of 17.4, 21.0 and 21.9 kD. These bands were not observed in the control hyphae or in hyphae shifted to 25°C. In addition, antibodies reacted positively with the major high molecular mass (72.4 kD) sclerotial polypeptide.

Antibodies raised against the major and minor sclerotial polypeptides of T. incarnata (W29) reacted positively (Fig. 55D) with a single polypeptide band of the W29 vegetative hyphae which had been shifted to 25°C for 14 days. This band was approximately 18 kD. Antibodies also reacted positively with a polypeptide band of identical molecular mass in the cultured sclerotia (Fig. 55D). Antibodies raised against the major and minor sclerotial polypeptides also reacted positively with a single polypeptide band in W29 control vegetative hyphae (Fig. 55D). However, this polypeptide was distinctly different and had a molecular mass of about 18.6 kD. Once again, the antibodies also reacted with a polypeptide of identical molecular mass in the cultured sclerotia (Fig. 55D). Anti W29 reacted with 3 distinct polypeptide bands in the mature sclerotia (Fig. 55D). A major band was labelled and had a molecular mass of about 17.0 kD. This band was not observed in either temperature treatments of the vegetative hyphae. The two other minor bands had molecular masses of

approximately 18.0 and 18.6 kD (Fig. 55D). These bands appeared to be differentially expressed in the vegetative hyphae depending on the temperature treatment.

5.4 DISCUSSION

Antibodies raised against the major sclerotial polypeptides provided a sensitive means of probing for the presence of these major polypeptides in the vegetative hyphae of snow molds. Western blotting has provided a means for evaluating the levels of major sclerotial polypeptides in vegetative hyphae exposed to different temperature regimes.

Russo et al. (1982) have shown, by Coomassie blue staining, that the major sclerotial protein in the mesophile, Sclerotinia sclerotiorum, is not present in vegetative hyphae. Similarly, in Chapter 2, it was also shown that the major sclerotial polypeptides did not appear to be present in the vegetative hyphae of the snow mold species examined. Results presented in this chapter using Western Blot analysis, a more sensitive assay (Eisen, 1980), have shown unequivocally that the major sclerotial polypeptide of M. borealis (W51) and C. psychromorbidus (LRS131) are not present in vegetative hyphae of these species growing at permissive temperature. However, when the same vegetative hyphae were shifted to 25°C for 14 days, the major sclerotial polypeptides were detected.

Since it has been shown that M. borealis (W51) and C. psychromorbidus (LRS131) accumulate major proteins as a function of sclerotial morphogenesis, the presence of the major sclerotial polypeptides in vegetative hyphae of these species exposed to high temperature may be indicative of the initiation of a temperature-induced differentiation process leading to the development of sclerotia. This observation provides the first biochemical support for temperature-regulated sclerotial morphogenesis in these species.

In contrast to M. borealis (W51) and C. psychromorbidus (LRS131), control hyphae of Typhula spp. showed evidence of one, or more sclerotial polypeptides. Thus, certain sclerotial polypeptides appeared to be constituitive in the vegetative hyphae of these Typhula species. This may indicate a) that these species have a different strategy for surviving nonpermissive temperatures or b) that these fungi maintain reduced levels of these major proteins in their vegetative hyphae in the event that sclerotial morphogenesis is required in a short period of time, or c) that low levels of these major proteins may be necessary for the purpose of sclerotial formation. At the moment, these explanations are speculative.

Furthermore, vegetative hyphae of T. idahoensis (W21) shifted to 25°C showed little or no change in the amount or size class of sclerotial polypeptide labelled, compared with the control treatment. The absence of certain

sclerotial polypeptides in the 17.0, 21.0 and 23.9 kD range and the lack of significant accumulation of other sclerotial polypeptides in the vegetative hyphae of T. idahoensis (W21), exposed to nonpermissive temperature treatments, may indicate a differential rate of protein turnover in this species compared to M. borealis (W51) and C. psychromorbidus (LRS131).

Vegetative hyphae of T. incarnata (W29) shifted to 25°C showed a distinct alteration in the size class of sclerotial polypeptide labelled. In the control hyphae, the labelled sclerotial polypeptide had a molecular mass of approximately 18.6 kD, whereas in hyphae shifted to 25°C, the labelled band had a molecular mass of approximately 18.0 kD. Thus, the temperature shift to 25°C caused a certain degree of variation in protein accumulation. However, there was no evidence for the accumulation of the major 17 kD sclerotial protein. At least two explanations are possible. A temperature-induced, post-translational cleavage of the 18.6 kD polypeptide normally expressed in the control hyphae may have occurred, or a totally new gene or set of genes was turned on due to the temperature increase to give rise to the 18.0 kD polypeptide. The lack of accumulation of the 17.0 kD major sclerotial polypeptide in vegetative hyphae of T. incarnata (W29), subjected to nonpermissive growth temperature treatments, may be due to a differential rate of protein turnover relative to M. borealis (W51) and C. psychromorbidus (LRS131). Clearly,

the two Typhula species appear to respond quite differently on exposure to nonpermissive growth temperatures than either M. borealis (W51) or C. psychromorbidus (LRS131). It is interesting to note that the two Typhula species are the least psychrophilic of the species examined (Newsted et al., 1985).

In the case of M. borealis (W51), antibodies raised against the major sclerotial polypeptide of cultured sclerotia were highly reactive and specific for the major sclerotial polypeptide found in both the cultured and field sclerotia of M. borealis. This observation confirms the results in Chapter 2 which indicated that the major sclerotial polypeptide found in cultured sclerotia of M. borealis was not an artifact of culturing conditions. It is also consistent with the results in Chapter 3 which indicated that proteins in crude extracts of cultured and field sclerotia of M. borealis (W51) were antigenically related. Reports in the literature regarding antibodies raised to major proteins in sclerotial-forming fungi have been sparse. This seems rather remarkable when considering the potential value of antibodies as specific probes for detecting major sclerotial proteins. Petersen et al. (1982) successfully used antibodies against major sclerotial proteins in mesophilic Sclerotinia spp. to examine antigenic relatedness in these fungi. However, they did not further exploit the antibodies by using Western blotting to study the accumulation of the major

sclerotial polypeptides in vegetative hyphae. Thus, this study represents one of the first to use Western blotting as a means to study sclerotial protein accumulation in vegetative hyphae of fungi.

CHAPTER 6

IN VIVO LABELLING AND FLUOROGRAPHIC ANALYSIS OF NEWLY SYNTHESIZED POLYPEPTIDES IN VEGETATIVE HYPHAE SUBJECTED TO DIFFERENT TEMPERATURE TREATMENTS

6.1 INTRODUCTION

In Chapter 5, the procedure of immunoblotting was used to probe for the presence of major sclerotial polypeptides in vegetative hyphae subjected to permissive and non-permissive growth temperature. Though these experiments enabled one to discern whether or not the major sclerotial polypeptides were present in the hyphae after the hyphae were subjected to permissive and nonpermissive temperature, they did not necessarily provide an estimate of when these polypeptides were synthesized. In this chapter, fluorographic analysis is used to examine newly synthesized polypeptides produced as a function of temperature treatment in vegetative hyphae. In addition, Western blotting is used to confirm whether or not these newly synthesized polypeptides are in fact the major sclerotial polypeptides. Dejardin and Ward (1971a) have shown that sclerotia of T. incarnata are produced in cultures shifted to nonpermissive temperatures. Since elevated temperature has been implicated as a possible stimulator of sclerotial development (Traquair et al., 1987), these studies should

prove useful.

6.2 MATERIALS AND METHODS

6.2.1 Kinetics of L-[^{35}S]methionine incorporation.

Vegetative hyphae of M. borealis (W51), grown at permissive temperature, were collected and transferred to glass homogenizer tubes containing 1 ml of incubation medium. The incubation medium consisted of BSM minus the agar (see Chapter 2) and non-labelled methionine at a concentration of 0.I µM. Hyphae were then collected as described in Chapter 5. A solution containing 75 µCi of L-[^{35}S]methionine (New England Nuclear, 1086 Ci/mmol) was added to the incubation medium such that the specific activity of the medium was 750 Ci/mmol methionine. The tubes were immediately placed at 10°C in a refrigerated water bath and incubated for 30, 60, 195, 360 and 720 minutes. This was done to provide an estimate of the time required for maximum L-[^{35}S]methionine incorporation.

At the end of the respective incubation times, the labelled media were discarded and the hyphae were rinsed twice with a cold, non-radioactive solution of 0.1 µM methionine. This removed excess label and effectively stopped L-[^{35}S]methionine incorporation.

The polypeptides were extracted and solubilized in the glass homogenizers (Radnoti) in 100 µl of an SDS extraction buffer containing proteolytic inhibitors as previously

described (Chapter 5). The homogenates were transferred to 1.5 ml microfuge tubes and centrifuged for 5 minutes at 14,000 xg. The supernatants were collected and divided into smaller aliquots. Protein concentration was determined as previously described using the method of Comings and Tack (1972). Samples were stored at -70°C until needed.

6.2.2 Determination of radioactive incorporation.

Radiolabelled samples were thawed and quickly vortexed to ensure homogeneity. A 5 µl aliquot was removed, added to 1 ml of 1N NaOH and incubated for 10 minutes at 37°C. The NaOH cleaves the tRNA-L-[^{35}S]methionine complexes so that the free L-[^{35}S]methionine can be washed through the filter (Clemens, 1984). In addition, a 5 µl aliquot of extraction buffer was taken and used as control to determine background radioactivity. After 10 minutes, 4 ml of ice cold 25% (w/v) TCA containing 20 µg/ml BSA as carrier was added to the reaction mixture. This mixture was incubated on ice for 30 minutes. All samples were filtered through Whatman 934-AH glass fibre filter discs under vacuum. The filters were washed twice with 10 ml of ice cold 8% (w/v) TCA. The filters were then washed again and dried under vacuum using 2 ml of acetone. The filters were transferred to scintillation vials containing 10 ml of Aquasol (New England Nuclear). Radioactivity was measured using a Beckman LS-230 Scintillation System.

6.2.3 Temperature treatments.

Vegetative hyphae from each species were subjected to 3 different temperature treatments. One batch of hyphae growing at permissive temperature (5°C) was collected and incubated in the labelled medium at 5°C. Therefore, there was no temperature shift involved in this treatment. This treatment served as the control. A second batch of hyphae growing at permissive temperature (5°C) was collected and incubated in the labelled medium at 10°C. Therefore, there was a temperature increase of 5°C involved in this treatment. A third set of plates containing vegetative hyphae grown at permissive temperature (5°C) was shifted to nonpermissive temperature (25°C) for 2 days prior to collection of the hyphae and incubation in the labelled medium at 10°C. In all treatments, hyphae were incubated in the labelled media containing L-[^{35}S]methionine (750 Ci/mmol) for 12 hours. After 12 hours, the hyphae were washed and the polypeptides extracted as described in the previous section. The homogenates were centrifuged and the supernatants were collected and divided into smaller aliquots. Labelled supernatants were stored at -70°C until used for Western blotting and/or fluorography.

6.2.4 One-dimensional SDS-PAGE separations of polypeptides.

One-dimensional SDS polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) with modifications. The separating gel consisted of a 12-

20% polyacrylamide linear gradient which was allowed to polymerize for 1 hour. A 5% (w/v) polyacrylamide stacking gel was poured on top of the separating gel and allowed to polymerize for 30 minutes. The labelled hyphal extracts were thawed and 1 µl of 0.5% (w/v) Bromophenol blue was added to each sample before they were placed in a boiling water bath for 1 minute. Samples consisting of either a constant amount of protein (0.8 µg) for Western blots or a constant amount of radioactivity (10,000 acid-precipitable counts) for fluorograms were routinely used. A 4 µl sample of standard proteins from a low molecular weight calibration kit (Pharmacia Fine Chemicals) was also added to one well for molecular mass determination of separated polypeptides. In addition, crude extracts of sclerotial proteins were added to another well and used as markers. The samples were electrophoresed at a constant current of 15 mA per gel using a Mighty Small II Slab Gel Electrophoresis Unit SE 250 (Hoefer Scientific Instruments) until the bromophenol blue dye ran off the end of the gel. This ensured that any free L-[^{35}S]methionine was electrophoresed off the gel. Gels loaded with constant amounts of protein were then removed and put through the Western blotting procedure described in Chapter 5. Gels loaded with equal counts were removed, stained for 10 minutes in a 0.1% solution of Coomassie brilliant blue R-250 in 40% methanol: 7.5% acetic acid and then destained in 40% methanol: 7.5% acetic acid.

6.2.5 Fluorographic detection of newly synthesized polypeptides.

Gels were prepared for fluorography as described by Bonner (1984). After destaining, the gels were immersed in four volumes of a 22% (w/v) solution of 2,5-diphenyloxazole (PPO) in acetic acid for 3 hours. They were then immersed in a gentle stream of tap water to precipitate the PPO within the gels. The gels were then transferred to a solution of 2% (v/v) glycerol in water for 10 minutes. This step prevents the gels from cracking during the drying procedure. The gels were then dried onto Whatman 3 MM filter paper with a Bio-Rad (Model 224) gel slab dryer. Fluorograms were prepared by apposing dried gels at -70°C to Kodak X-Omat AR film which was preflashed to an optical density of 0.15 (Laskey and Mills, 1975).

The exposed films were developed in Kodak GBX developer and replenisher for 5 minutes at 20°C, rinsed with tap water for 30 seconds, fixed with Kodak GBX fixer and replenisher for 2-4 minutes at 20°C, washed in running tap water for 5 minutes and then hung to air dry. Fluorograms were photographed on a light box using Kodak 2415 Technical Pan film in a 35 mm camera.

6.3 RESULTS

6.3.1 Kinetics of L-[^{35}S]methionine incorporation.

In vegetative hyphae of M. borealis (W51), the amount of L-[^{35}S]methionine incorporated into TCA precipitable

material steadily increased until 6 hours of incubation at 10°C (Fig. 56). Only a marginal increase was observed between 6 and 12 hours. Therefore, it was concluded that maximum incorporation had occurred by 12 hours under these incubation conditions. Thus, all subsequent incubations of vegetative hyphae were allowed to proceed for 12 hours.

6.3.2 Temperature treatments.

Fluorographic analysis of newly synthesized polypeptides in vegetative hyphae of M. borealis (W51) indicated that little or no labelling of the major sclerotial polypeptide occurred during incubation at 5°C (Fig. 57, lane 1). This was confirmed by the corresponding Western blot of the same polypeptide profile. Antibodies raised against the major sclerotial polypeptide of M. borealis (W51) showed no reaction with any polypeptide bands in vegetative hyphae of W51 which were incubated at 5°C (Fig. 57, lane 4). In contrast, fluorographic analysis of newly synthesized polypeptides in vegetative hyphae of M. borealis (W51), incubated at 10°C in the presence of L-[^{35}S]methionine, indicated that there was copious labelling of a predominant polypeptide of approximately 33.9 kD (Fig. 57, lane 2). Western blot analysis confirmed that this major polypeptide was the major sclerotial polypeptide (Fig. 57, lane 5). However, fluorographic analysis showed no labelling of the major (33.9 kD) polypeptide (Fig. 57, lane 3) when vegetative hyphae of M.

Figure 56. Plot showing incorporation of radioactive methionine into protein as a function of time in vegetative hyphae of M. borealis (W51).

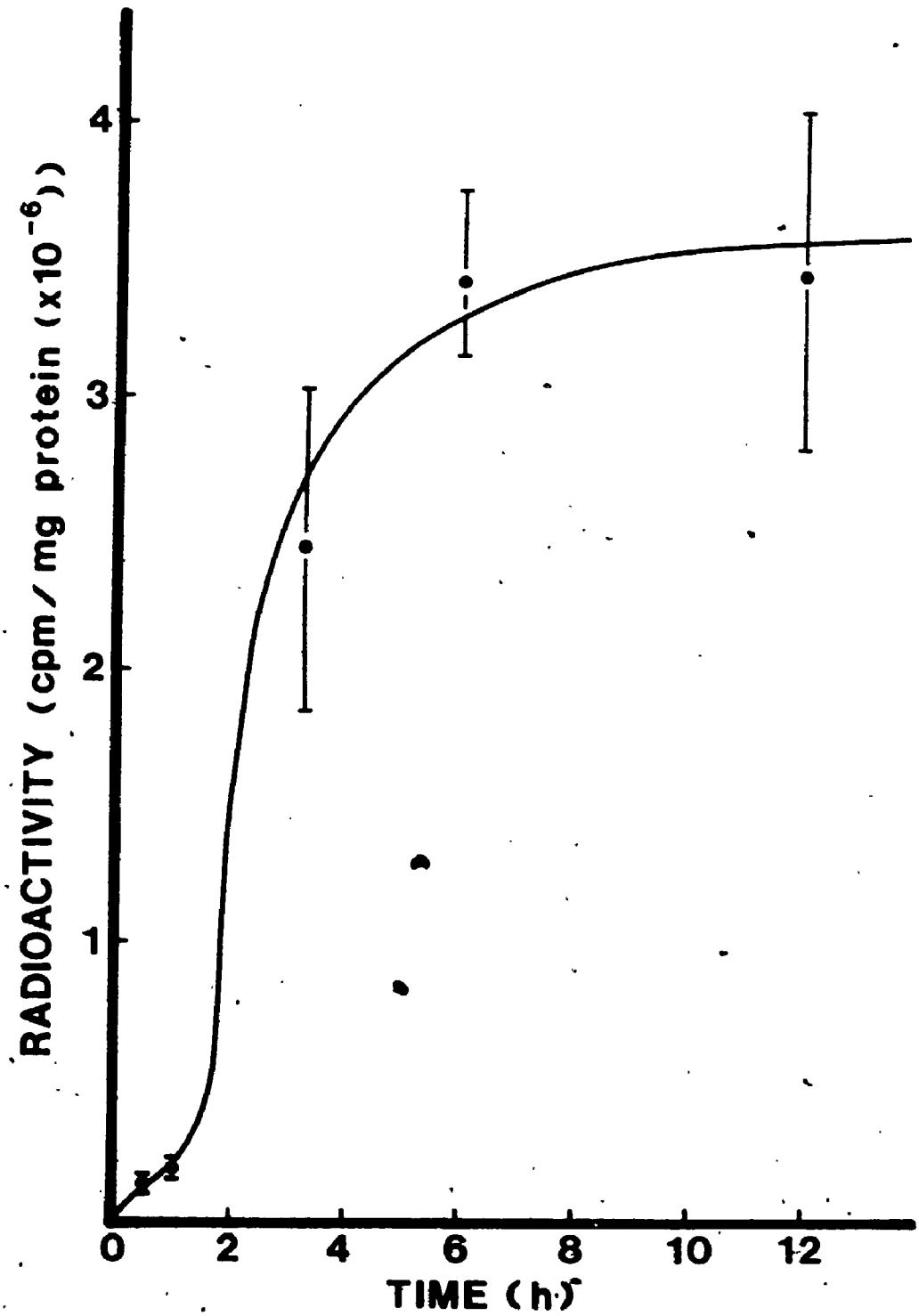
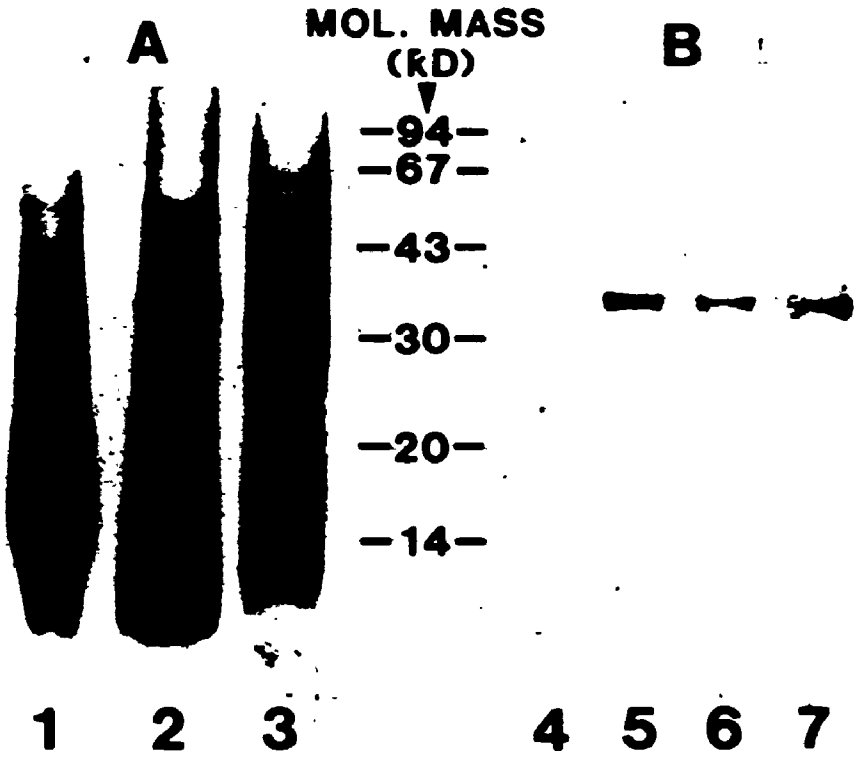

RADIOACTIVITY (cpm / mg protein (x10⁻⁶))
0
1
2
3
4
0
2
4
6
8
10
12
TIME (h)

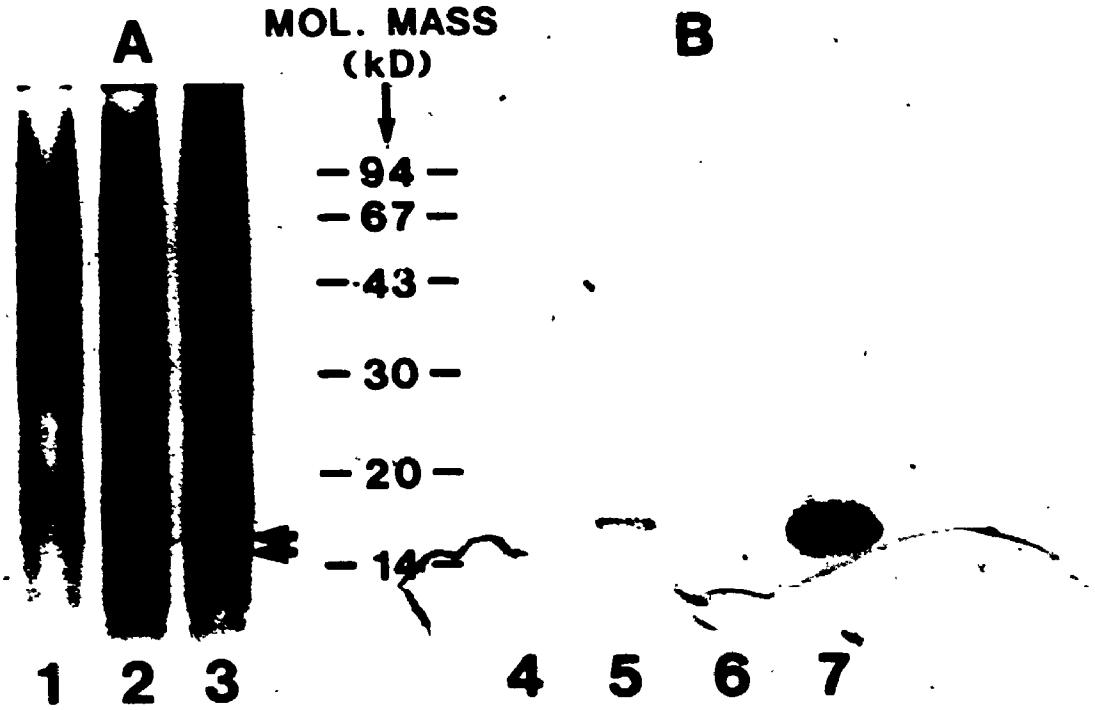
Figure 57. Fluorography and Western blot analysis of newly synthesized polypeptides in vegetative hyphae of M. borealis (W51). (A) fluorogram: lanes 1 and 2, newly synthesized polypeptides of vegetative hyphae incubated at 5°C and 10°C respectively in the presence of L-[^{35}S]methionine; lane 3, newly synthesized polypeptides of vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C. (B) Western blot probed with antiserum raised to the major sclerotial polypeptide of M. borealis (W51): lanes 4 and 5, vegetative hyphae incubated at 5°C and 10°C respectively; lane 6, vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C; lane 7, mature sclerotia. Molecular weight standards are indicated in the centre.


A
MOL. MASS
(kD)
B
94
67
43
30
20
14
1
2
3
4
5
6
7

borealis (W51) was shifted to 25°C for 2 days prior to incubation at 10°C in the presence of L-[^{35}S]methionine. Comparison of the corresponding Western blot (Fig. 57, lane 6) indicated that the major sclerotial polypeptide was present in these vegetative hyphae as denoted by the single labelled 33.9 kD polypeptide band.

Fluorographic analysis of newly synthesized polypeptides in vegetative hyphae of C. psychromorbidus (LRS131) indicated that little or no labelling of the major (12.9-14.5 kD) sclerotial polypeptides occurred during incubation at 5°C (Fig. 58, lane 1). This was also confirmed by the corresponding Western blot of the same polypeptide profile. Antibodies raised against the major sclerotial polypeptides of C. psychromorbidus (LRS131) showed no reaction with any polypeptide bands in vegetative hyphae of LRS131 which were incubated at 5°C (Fig. 58, lane 4). However, vegetative hyphae of C. psychromorbidus (LRS131) incubated at 10°C in the presence of L-[^{35}S]methionine, and vegetative hyphae of C. psychromorbidus (LRS131) shifted to 25°C for 2 days prior to incubation at 10°C in the presence of L-[^{35}S]methionine both showed labelling of polypeptides with molecular weights corresponding to that of the major sclerotial polypeptides of C. psychromorbidus (LRS131) (Fig. 58, lanes 2 and 3). This was especially evident in vegetative hyphae of C. psychromorbidus (LRS131) shifted to 25°C for 2 days (Fig. 58, lane 3, arrows). Western blots of the

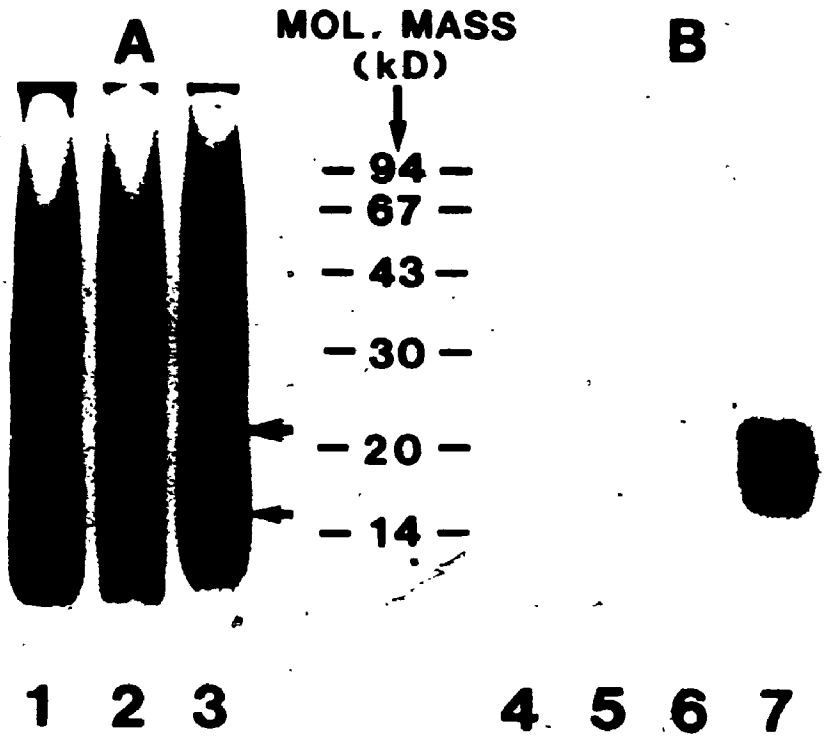
Figure 58. Fluorography and Western blot analysis of newly synthesized polypeptides in vegetative hyphae of C. psychromorbidus (LRS131). (A) fluorogram: lanes 1 and 2, newly synthesized polypeptides of vegetative hyphae incubated at 5°C and 10°C respectively in the presence of L-[^{35}S]methionine; lane 3, newly synthesized polypeptides of vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C; arrows indicate major sclerotial polypeptides. (B) Western blot probed with antiserum raised to the major sclerotial polypeptides of C. psychromorbidus (LRS131): lanes 4 and 5, vegetative hyphae incubated at 5°C and 10°C respectively; lane 6, vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C; lane 7, mature sclerotia. Molecular weight standards are indicated in the centre.


A
MOL. MASS
(kD)
B
94
67
43
30
20
14
1 2 3 4 5 6 7

radiolabelled polypeptides of vegetative hyphae of C. psychromorbidus (LRS131) incubated at 10°C and hyphae shifted to 25°C for 2 days verified that the major sclerotial polypeptides were present in these hyphae (Fig. 58, lanes 5 and 6). However, detection of the major sclerotial polypeptides was more obvious using the Western blot procedure than the radiolabelling procedure. This was observed by comparing Fig. 58, lanes 2 and 3, with Fig. 58, lanes 5 and 6.

In contrast to the results for M. borealis (W51) and C. psychromorbidus (LRS131), fluorographic analysis of newly synthesized polypeptides in vegetative hyphae of T. idahoensis (W21) indicated that very low levels of synthesis of the major low molecular weight sclerotial polypeptides did occur in vegetative hyphae incubated at 5°C in the presence of L-[^{35}S]methionine (Fig. 59, lane 1). In these hyphae, the most prominent labelled polypeptide in the low molecular weight class was approximatley 17.0 kD (Fig. 59, lane 1). This was also observed in vegetative hyphae incubated at 10°C (Fig. 59, lane 2). In addition, there was also evidence of very low levels of labelling of the major sclerotial polypeptide which corresponded to the 21.9 kD sclerotial polypeptide (Fig. 59, lanes 1 and 2). These observations were consistent with the corresponding Western blot data where antibodies, raised against the major low molecular mass sclerotial polypeptides, reacted positively with major sclerotial polypeptide bands in the

Figure 59. Fluorography and Western blot analysis of newly synthesized polypeptides in vegetative hyphae of T. idahoensis (W21). (A) fluorogram: lanes 1 and 2, newly synthesized polypeptides of vegetative hyphae incubated at 5°C and 10°C respectively; lane 3, newly synthesized polypeptides of vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C; upper and lower arrows indicate the 21.9 and 15 kD polypeptides. (B) Western blot probed with antiserum raised to the major low molecular mass sclerotial polypeptides of T. idahoensis (W21): lanes 4 and 5, vegetative hyphae incubated at 5°C and 10°C respectively; lane 6, vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C; lane 7, mature sclerotia. Molecular weight standards are indicated in the centre.


A
MOL. MASS
(kD)
B
94
67
43
30
20
14
1 2 3
4 5 6 7

17.0 kD and 21.9 kD range (Fig. 59, lanes 4 and 5), revealing very low levels of these polypeptides.

However, fluorographic analysis of newly synthesized polypeptides in vegetative hyphae of T. idahoensis shifted to 25°C for 2 days prior to labelling at 10°C showed that there was a distinct increase in the labelling of polypeptides in the 21.9 and 15 kD range (Fig. 59, lane 3, upper and lower arrows respectively). The corresponding Western blot showed that antibodies, raised against the major low molecular mass sclerotial polypeptides, reacted positively with the 21.9 kD band, verifying that it was a major sclerotial polypeptide (Fig. 59, lane 6). However, Western blot analysis did not detect the 15 kD polypeptide.

Fluorographic analysis of labelled polypeptides in vegetative hyphae of T. incarnata (W29) indicated that approximately equal amounts of labelling of two minor sclerotial polypeptides occurred in vegetative hyphae incubated at 5°C and 10°C in the presence of L-[^{35}S]methionine (Fig. 60, lanes 1 and 2). This was confirmed by the corresponding Western blot where antibodies raised against the major and minor sclerotial polypeptides of T. incarnata (W29) reacted positively against these sclerotial polypeptides in vegetative hyphae incubated at 5°C and at 10°C (Fig. 60, lanes 4 and 5). However, according to the fluorogram, there was little or no labelling of the major (17.0 kD) sclerotial polypeptide.

In contrast, it was found that transfer of the

Figure 60. Fluorography and Western blot analysis of newly synthesized polypeptides in vegetative hyphae of T. incarnata (W29). (A) fluorogram: lanes 1 and 2, newly synthesized polypeptides of vegetative hyphae incubated at 5°C and 10°C respectively; lane 3, newly synthesized polypeptides of vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C. (B) Western blot probed with antiserum raised to the major and minor sclerotial polypeptides of T. incarnata (W29): lanes 4 and 5, vegetative hyphae incubated at 5°C and 10°C respectively; lane 6, vegetative hyphae shifted to 25°C for 2 days prior to labelling at 10°C; lane 7, mature sclerotia. Molecular weight standards are indicated in the centre.


A
MOL. MASS
(kD)
B
-94-
-67-
-43-
-30-
-20-
-14-
1 2 3 4 5 6 7

vegetative hyphae of T. incarnata (W29) to 25°C, prior to incubation at 10°C in the presence of L-[^{35}S]methionine, had a profound effect on the synthesis of newly synthesized polypeptides. First, fluorographic analysis indicated that labelling of the two minor sclerotial polypeptides had nearly ceased (Fig. 60, lane 3). Secondly and more importantly, copious labelling of a new polypeptide of approximately 35 kD occurred (Fig. 60, lane 3). Western blot analysis using antibodies raised against the major and minor sclerotial polypeptides of T. incarnata (W29) reacted positively, though weakly, with a polypeptide of this molecular mass (Fig. 60, lane 6). In addition, Western blotting indicated that the major and minor sclerotial polypeptide bands were present but at low levels (Fig. 60, lane 6). It appeared, therefore, that this new 35 kD polypeptide was antigenically related to at least one of the major or minor sclerotial polypeptides.

6.4 DISCUSSION

The combination of fluorographic analysis and Western blot analysis provided a powerful tool for examining gene expression in the vegetative hyphae of psychrophilic fungi. In vivo labelling enabled the observation of newly synthesized polypeptides in response to temperature, while Western blotting confirmed the identity of the newly synthesized polypeptides as the major sclerotial

polypeptides.

Results clearly indicated that there was no constituitive expression of the major (33.9 kD) sclerotial polypeptide of M. borealis (W51) in vegetative hyphae grown at permissive temperature (5°C). This was confirmed by both fluorography and Western blotting. However, when vegetative hyphae of M. borealis (W51) were transferred from 5°C and incubated at 10°C in the presence of L-[^{35}S]methionine, synthesis of the major (33 kD) sclerotial polypeptide was induced. Thus a small increase in incubation temperature of 5°C over 12 hours was sufficient to cause synthesis of the major (33 kD) sclerotial polypeptide in vegetative hyphae of M. borealis (W51). Both fluorography and Western blotting confirmed this. Furthermore, expression of this 33.9 kD sclerotial polypeptide in vegetative hyphae of M. borealis (W51) appeared to be the most temperature-sensitive system. This is consistent with the results of Newsted et al. (1985) where vegetative growth of M. borealis (W51) was shown to be the most temperature sensitive. Expression of major sclerotial polypeptides in vegetative hyphae of the other snow mold species were not as temperature sensitive. This is consistent with recent data of Newsted et al. (1985) which showed that growth of vegetative hyphae of M. borealis (W51) was more sensitive to nonpermissive temperatures than the Typhula species.

In addition, shifting hyphae of M. borealis (W51) to

25°C for 2 days prior to labelling appeared to arrest the synthesis of the major (33.9 kD) sclerotial polypeptide. However, corresponding Western blot data indicated that the major sclerotial polypeptide was still present in these hyphae. One possible explanation for these results would be that a sudden and massive pulse of synthesis of the 33.9 kD sclerotial polypeptide occurred just prior to labelling. However, a more probable explanation would be that synthesis of the major (33.9 kD) sclerotial polypeptide occurred earlier during the two day shift to 25°C, followed by a subsequent cessation of synthesis prior to labelling. If proteolysis of the major (33.9 kD) sclerotial polypeptide was occurring during the period after which this polypeptide was synthesized, then it was not severe or rapid enough to cause a total degradation of this polypeptide. This is consistent with the results described in Chapter 5 where the major sclerotial polypeptide was still detected by Western blot analysis of protein extracts from vegetative hyphae which had been shifted to 25°C for two weeks. Therefore, these results suggest that the major sclerotial polypeptide of M. borealis (W51) may be resistant to proteolysis at 25°C. It is also interesting that vegetative hyphae shifted to 25°C for two days and then incubated at 10°C for 12 hours in the presence of L-[^{35}S]methionine, exhibited little or no recovery of synthesis of the major sclerotial polypeptide. Furthermore, synthesis of all polypeptides was terminated

in vegetative hyphae of M. borealis (W51) shifted to 25°C for 2 weeks, and no recovery of synthesis was observed when the hyphae were incubated at 10°C for 12 hours in the presence of L-[^{35}S]methionine (data not shown). This indicates that incubation of vegetative hyphae of M. borealis (W51) at 25°C can be lethal if the period of incubation is long enough.

Little or no expression of the major (12.9-14.5 kD) sclerotial polypeptides of C. psychromorbidus (LRS131) was observed in vegetative hyphae incubated at 5°C. This was verified by fluorography and Western blotting. Hyphae incubated at 10°C in the presence of L-[^{35}S]methionine showed faint expression of the major sclerotial polypeptides according to fluorographic analysis, whereas the corresponding Western blot indicated that considerable amounts of the major sclerotial polypeptides were present in these hyphae. Similar results were observed for hyphae shifted to 25°C for two days prior to labelling. A low methionine content in the major (12.9-14.5 kD) sclerotial polypeptides of LRS131 may be a possible explanation to account for this apparent discrepancy.

Vegetative hyphae of T. idahoensis (W21) and T. incarnata (W29) incubated at permissive temperature appeared to synthesize constituitively low levels of certain, but not all sclerotial polypeptides. This is in direct contrast to vegetative hyphae of M. borealis (W51) and C. psychromorbidus (LRS131) which do not synthesize

constituitively sclerotial polypeptides when growing at permissive temperature. The reason for constituitive expression of sclerotial polypeptides at permissive temperatures in the Typhula species is unknown. However, it may somehow reflect a difference in survival strategy.

Also, incubating the hyphae of the Typhula species at 10°C instead of 5°C while radiolabelling had little effect on synthesis, as seen by fluorography, and on the amount of sclerotial polypeptides accumulated, as seen by Western blotting. Thus, the regulation of sclerotial polypeptide synthesis in the vegetative hyphae of the Typhula species appeared to be relatively insensitive to small temperature increases. Again, this contrasts with the sensitivity to small temperature increases observed in the vegetative hyphae of M. borealis (W51) and C. psychromorbidus (LRS131).

Though constituitive expression of the major sclerotial polypeptides in vegetative hyphae of T. idahoensis (W21) and T. incarnata (W29) was relatively unaffected by a small increase in incubation temperature from 5°C to 10°C, sclerotial polypeptide expression in vegetative hyphae of these species was markedly influenced by a two day shift to nonpermissive temperature prior to labelling. Vegetative hyphae of T. idahoensis (W21) shifted to 25°C for two days prior to labelling showed a distinct enhancement of synthesis of the sclerotial polypeptide in the 72 kD range.

In contrast, vegetative hyphae of T. incarnata (W29)

shifted to 25°C for two days prior to labelling showed a distinct reduction in synthesis of the minor sclerotial polypeptide bands, but more importantly, there was a sudden and massive synthesis of a polypeptide of approximately 35 kD. Furthermore, this 35 kD polypeptide appears to be antigenically related to at least one of the major or minor sclerotial polypeptides as shown by supporting Western blot analysis. Whether this 35 kD polypeptide is a precursor of one of the sclerotial polypeptides is unknown at this time. It is also possible that the 35 kD polypeptide represents a type of heat shock protein since heat shock proteins in the 30-38 kD range have been reported in fungi (Plesofsky-Vig and Brambl, 1985a). However, most heat shock proteins in fungi are synthesized for only 30-60 minutes immediately after temperature shift (Plesofsky-Vig and Brambl, 1985a), whereas the 35 kD polypeptide is obviously still synthesized after 48 hours of incubation at nonpermissive temperature. In this way, the 35 kD polypeptide is therefore atypical of fungal heat shock proteins.

It is also interesting that the high (72.4 kD) molecular mass sclerotial polypeptide of T. idahoensis (W21) was not detected via fluorography or Western blot analysis in vegetative hyphae during any of the temperature treatments. This was surprising since a polypeptide of 72.4 kD was shown to be present in sclerotial initials (Chapter 2). It was postulated in Chapter 1 that this 72.4 kD polypeptide could be a precursor which was post-

translatically cleaved to yield the major low (16.0-21.9 kD) molecular mass sclerotial polypeptides. However, these studies, though preliminary, present no evidence for temperature induced synthesis of the 72.4 kD polypeptides. Three explanations are proposed: 1) either high temperature shift does not induce expression of the 72.4 kD polypeptide; or 2) High temperature shift alone may not be sufficient to induce expression of this polypeptide; or 3) expression of this polypeptide may require longer periods of incubation at nonpermissive temperature than was provided in these experiments. Clearly more work needs to be done in order to determine what induces synthesis of this polypeptide.

At this point, it can be concluded that vegetative hyphae of M. borealis (W51) and C. psychromorbidus (LRS131) can be induced by high temperature shift to synthesize the major sclerotial polypeptides. Of the two species, expression of the major sclerotial polypeptides in vegetative hyphae of M. borealis (W51) appears to be the most sensitive to high temperature as evidenced by a complete cessation of synthesis of the major sclerotial polypeptides after two days at 25°C. These polypeptides do not appear to be "heat shock" proteins since synthesis of these major sclerotial polypeptides is specifically linked to sclerotial development. It may be more appropriate to call them "developmental proteins", the expression of which can be temperature regulated. It is therefore tempting to

conclude that temperature is a factor regulating sclerotial development. This hypothesis is supported by the work of Dejerdin and Ward (1971a) where it was shown that sclerotia in three Typhula species were produced more abundantly at higher growth temperatures. Very recently, Traquair et al. (1987) also showed that sclerotial production was stimulated in C. psychromorbidus when cultures were subjected to supraoptimal growth temperatures. This phenomenon was also observed in the species examined in this study (data not shown). Furthermore, it has been shown in field sclerotia of M. borealis and in cultured sclerotia of C. psychromorbidus (LRS131) that the final destination of these major sclerotial polypeptides is in protein bodies (Chapter 4). This suggests strongly that these polypeptides may function as storage proteins to be later used for carpogenic germination (Russo and Van Etten, 1985).

It has also been shown from these studies that the Typhula species express constituitively certain sclerotial polypeptides. This would suggest that temperature is not as crucial a stimulus in regulating expression of these major sclerotial polypeptides in vegetative hyphae of these species as compared to M. borealis (W51) and C. psychromorbidus (LRS131). It is also important to note that subjecting the hyphae of T. idahoensis (W21) to nonpermissive temperature appeared to enhance the synthesis of at least one (22 kD) polypeptide. Thus, a certain

degree of temperature regulated synthesis of the major sclerotial polypeptides was demonstrated in vegetative hyphae of T. idahoensis (W21). In contrast, vegetative hyphae of T. incarnata (W29) shifted to 25°C for two days prior to labelling caused a significant reduction in the synthesis of the minor sclerotial polypeptide bands and a concomitant massive increase in synthesis of a 35 kD polypeptide.

Presently, it is not known at what level synthesis of the major sclerotial polypeptides is regulated in the psychrophilic sclerotial-forming fungi. As demonstrated for heat shock proteins in fungi (Plesofsky-Vig and Brambl, 1985a), expression of these polypeptides could be regulated at the level of transcription, post-transcription, translation or post-translation. Clearly, more work needs to be done in this regard.

CHAPTER 7

A SUMMARY

7.1 MAJOR CONCLUSIONS

I have attempted to provide insights on major polypeptides associated with sclerotial development in the four psychrophilic fungi, M. borealis, C. psychromorbidus, T. idahoensis and T. incarnata. The data presented in Chapter 2 represent the first identification and characterization of major polypeptides associated with sclerotial development in psychrophilic species. It was shown in Chapter 3 that immunological relatedness exists among some of the major sclerotial polypeptides of these species. This may be indicative of a common role or function for these polypeptides. In Chapter 4, it was demonstrated that these major sclerotial polypeptides were localized in protein bodies much like those found in the seeds of plants, thus indicating a possible storage role for these polypeptides. Furthermore, the immunochemical studies presented in Chapter 4 represent the first report of the localization of major sclerotial polypeptides in psychrophilic fungi. These studies also provide the first confirmation of the work of Russo and Van Etten (1985) who showed that the major sclerotial proteins of S. sclerotiorum were also sequestered in protein bodies. Data

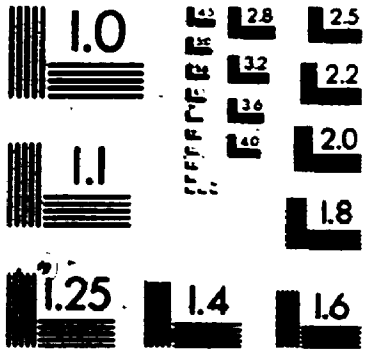
presented in Chapter 5 show for the first time that the major sclerotial polypeptides of M. borealis and C. psychromorbidus are not expressed constituitively in vegetative hyphae growing at permissive temperature, whereas constituitive expression was detected in the Typhula species. In addition, nonpermissive temperature appeared to induce the accumulation of the major sclerotial polypeptides in certain pecies. Lastly, in Chapter 6, it was shown for the first time that different temperatures can have a profound influence on the synthesis of the major sclerotial polypeptides in vegetative hyphae of sclerotial-forming snow molds. Moreoever, results in this chapter provide the first evidence for temperature regulated synthesis of major polypeptides associated with sclerotial development in these psychrophiles. Clearly, the observations presented in Chapter 6 reveal the real potential for studying the temperature regulation of sclerotial development at the molecular level, using the sclerotial polypeptides as markers for gene expression.

7.2 FUTURE WORK

In Chapter 4, it was clearly demonstrated that the major sclerotial polypeptides were sequestered in protein bodies. Though this procedure enabled the localization of the major sclerotial polypeptides after they were sequestered in large, conspicuous protein bodies, it did

not enable the fine ultrastructural observation required for localizing the site of protein synthesis, nor did it enable the observation of the process of protein packaging. Questions regarding these processes could probably be answered using electron microscopy and protein A-gold labelling of embedded sections of sclerotial initials probed with the primary antisera raised in this study. This assay is much more sensitive and should facilitate observations at the ultrastructural level.

The work presented in Chapter 6 has demonstrated that thermal shifts can induce synthesis of the major sclerotial polypeptides in the vegetative hyphae of snow molds. This raises the question as to whether the genes coding for the major sclerotial polypeptides are being regulated at the transcriptional or translational level. Lewin (1983) has noted that sporulation in bacteria involves a drastic change in the biosynthetic activities of the bacterium and that a large number of genes are involved. He also noted that the basic level of control was at the level of transcription. It would be interesting to determine if this was true in sclerotial-forming snow molds. If regulation is occurring at the translational level, then in vitro translation of the isolated mRNA from vegetative hyphae growing at 5°C (permissive temperature) should result in the expression of the major sclerotial polypeptides. If the major sclerotial polypeptides are not expressed by in vitro translation, then regulation is



probably occurring at the transcriptional level. Such studies should provide more insight into the regulation of gene expression during sclerotial development in the psychrophilic sclerotial-forming fungi.

APPENDIX I. Vitamin solution used for preparing BSM according to Hannay (1967).

VITAMIN SOLUTION

Vitamin	Concentration (g/l)
Adenylic acid	0.1
D-biotin	0.01
Choline chloride	0.2
Inosine	0.268
Nicotinamide	0.5
Pyridoxal hydrochloride	0.2
Pyridoxamine dihydrochloride	0.2
Thiamine hydrochloride	0.2
Riboflavin	0.02

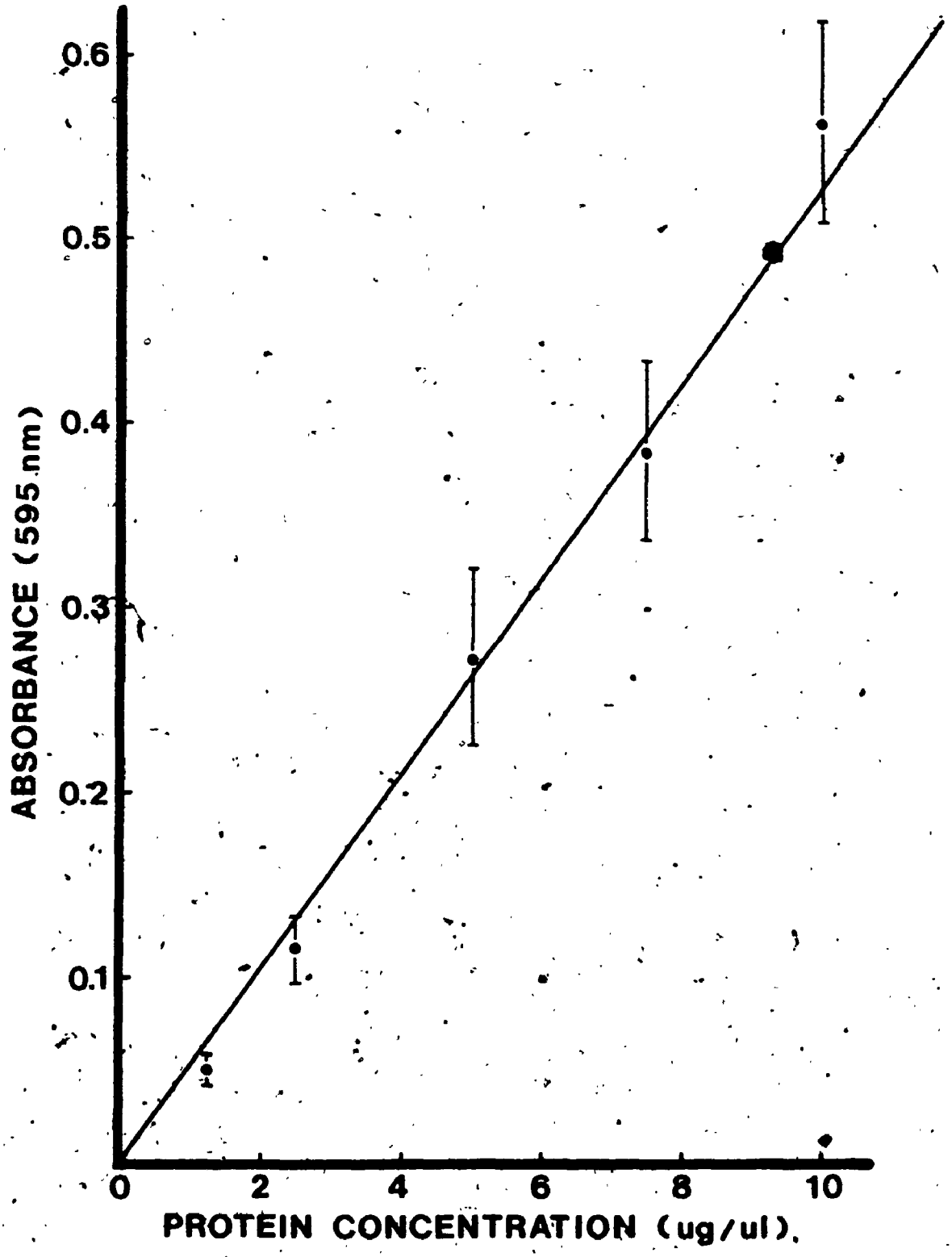
APPENDIX II. Minor nutrient solution used for preparing BSM according to Ward and Colotelo (1960) with modifications.

NUTRIENT SOLUTION

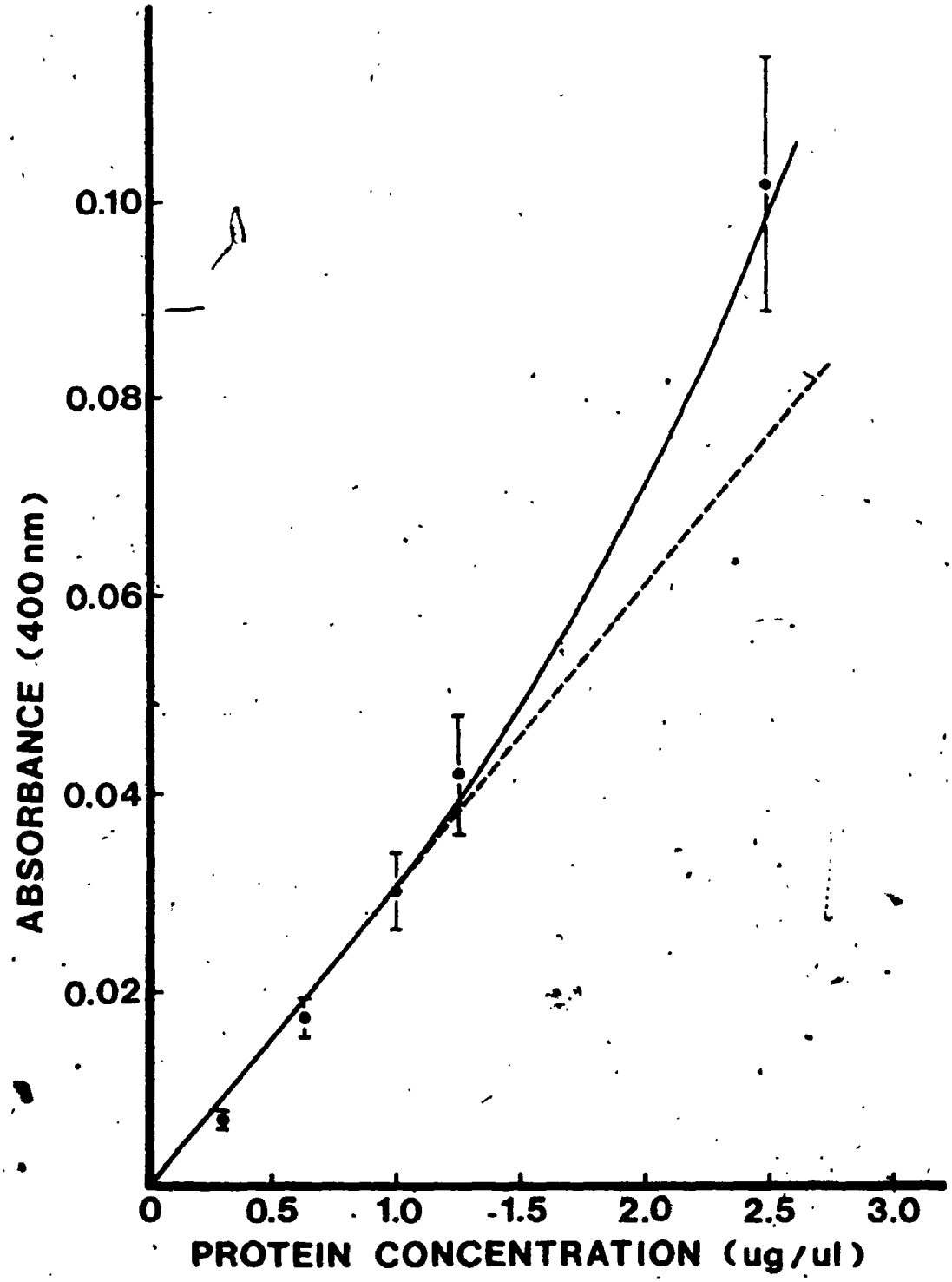
Nutrient	Concentration (g/l)
$Zn SO_4 . 7H_2O$	0.961
$CoCl_2 . 6H_2O$	0.245
$MnCl_2 . 4H_2O$	0.135
H_2MoO_4	0.0765
$CuSO_4 . 5H_2O$	0.195
$CaCl_2 . 2H_2O$	5.297
$*Fe_2(SO_4)_3$	0.715

* Added as a separate solution chelated with equimolar amounts of EDTA.

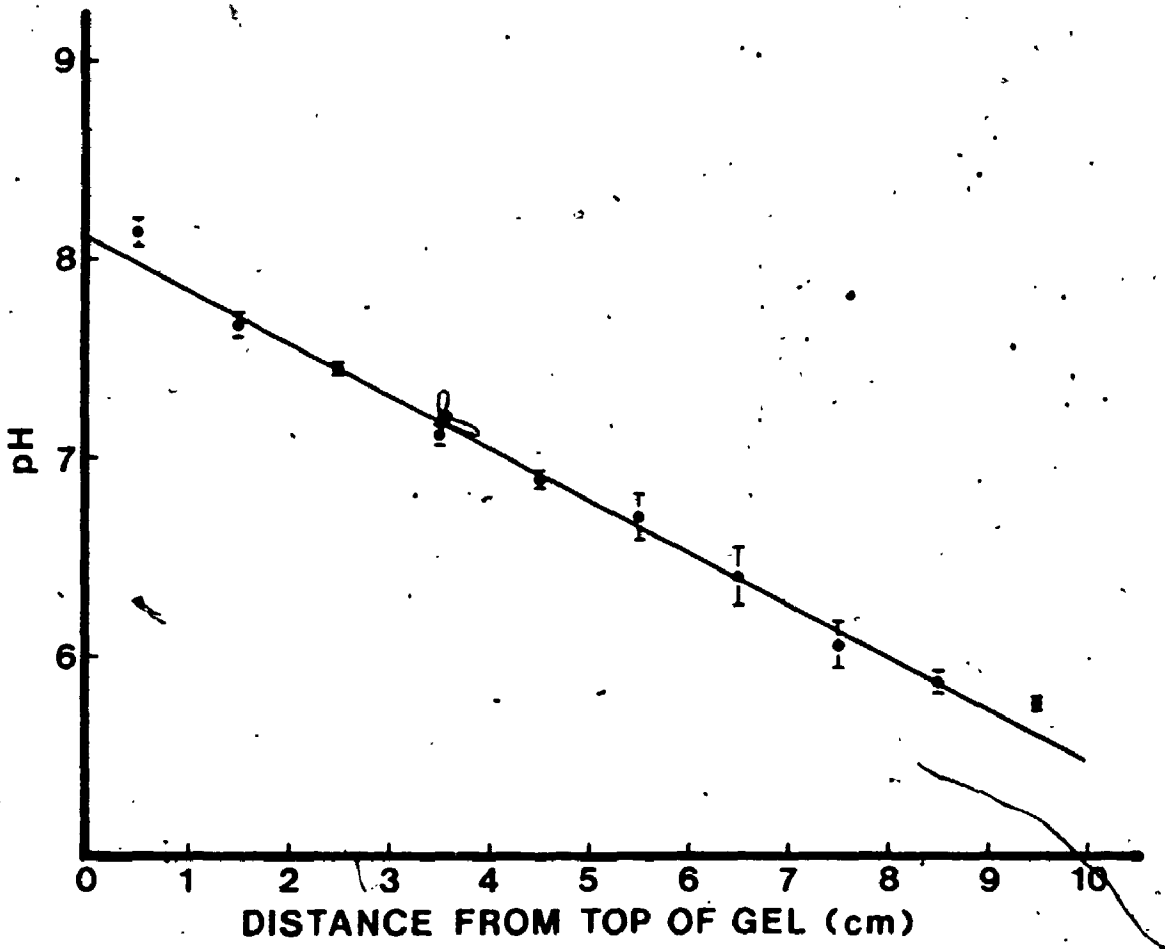
APPENDIX III. Standard curve used for estimating protein concentrations according to the method of Bradford (1976). Protein concentration using BSA as the standard is plotted against absorbance at 595 nm. The linearity of the curve is indicated. Each point represents the mean value for five determinations. Bars indicate standard deviation.


ABSORBANCE (595 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
PROTEIN CONCENTRATION (ug/ul).

APPENDIX IV. Standard curve used for estimating protein concentrations according to the method of Comings and Tack (1972). Protein concentration using BSA as the standard is plotted against absorbance at 400 nm. Curves tend to deviate from linearity at concentrations higher than 1.5 µg/µl. This is probably due to light scattering. Each point represents the mean value for five determinations. Bars indicate standard deviation.


0.10
0.08
0.06
0.04
0.02
ABSORBANCE (400 nm)
0
0.5
1.0
1.5
2.0
2.5
3.0
PROTEIN CONCENTRATION (ug/ul)

APPENDIX V. Linear pH gradient established by plotting pH values against distance along the length of a focussed IEF tube gel. Ampholines used in the IEF gels are described in the Methods section of Chapter 2. The points represent the mean values for determinations made on four IEF gels. Bars indicate standard deviation.


9
8
7
6
pH
0
1
2
3
4
5
6
7
8
9
10
DISTANCE FROM TOP OF GEL (cm)

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